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UNIVERSITY OF ALBERTA

STRUCTURAL AND FUNCTIONAL STUDIES OF HUMAN AND INSECT LIPOPROTEINS

by

© Hu Liu

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

DEPARTMENT OF BIOCHEMISTRY

Edmonton, Alberta
FALL, 1993

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Canadä

University
of
Alberta

Lipid and Lipoprotein Research Group
Faculty of Medicine

328 Heritage Medical Research Centre
Edmonton, Alberta, Canada T6G 2S2

Telephone (403) 492-1333
Fax (403) 492-3383

Departmental
Affiliation

Biochemistry

Luis B. Agellon
(403) 492-5251

David N. Brindley
(403) 492-2078

Robert O. Ryan
(403) 492-5153

Dennis E. Vance
(403) 492-8286

Zemin Yao
(403) 492-9193

Medicine

Jean E. Vance
(403) 492-7250

Shinji Yokoyama
(403) 492-2963

Pediatrics

Gary D. Lopaschuk
(403) 492-2170

Physiology

Mark J. Poznansky
(403) 492-5074

Associate
Member

Pediatrics

Leslie J. Smith
(403) 492-3339

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Department of Biochemistry
University of Alberta
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Hu Liu

Hu Liu
Lipid and Lipoprotein Research Group
Department of Biochemistry
University of Alberta
Edmonton, Alberta, Canada
T6G 2S2

14 September, 1993

山重水复疑无路
柳暗花明又一村

Countless big mountains and deep rivers ahead,
I wondered where the path is?
Passed huge dark woodlands and seeing many blossoming flowers,
I found another village of new lives.

-----Quoted from a famous Chinese poem to describe the frustration and excitement of scientific research

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled STRUCTURAL AND FUNCTIONAL STUDIES OF HUMAN AND INSECT LIPOPROTEINS submitted by Hu Liu in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Robert O. Ryan

Dr. Robert O. Ryan (Supervisor)

Cyril M. Kay

Dr. Cyril M. Kay

Brian D Sykes

Dr. Brian D. Sykes

Dr. Shinji Yokoyama

Dr. Norbert H. Haunerland (External Examiner)

Dated: September 10, 1993

Dedication to:

My parents, Quanxiao & Xiuying

My wife, Lili

My children, Rebecca & Bill

For their love, support and patience

Summary

Lipoprotein structures from both mammalian and invertebrate sources have been studied by a variety of biochemical approaches.

In insect oocytes very high density lipophorin (VHDLp-E) is derived from adult moth hemolymph high density lipophorin (HDLp-A). Such transformation is catalyzed by a lipid transfer particle (LTP) present inside the oocyte. The same LTP was found in hemolymph. Incubation of human low density lipoprotein (LDL) with insect lipoprotein and high density lipophorin (HDLp) resulted in a net lipid transfer between the two lipoprotein particles in the presence of LTP. The loss of core lipid, diacylglycerol, decreases the demands on surface components, causing the dissociation of surface amphipathic apolipophorin III (apoLp-III) which originally associated with HDLp-A. Lipid transfer particle could also be used to transform several human high density lipoprotein (HDL) particles into an LDL-size particle with a reduced surface to volume ratio, leading to the dissociation of amphipathic apolipoprotein A-I (apo A-I), a by-product isolated without denaturing the HDL particles. Having a higher lipid binding affinity than apoLp-III, apo A-I could displace apoLp-III molecules present on the surface of the low density lipophorin (LDLp) particle. Lipid enriched LDLp particles are stabilized by binding an additional 14 apoLp-III molecules. Lipid transfer particle present in the hemolymph as well as other tissues functions in the lipid loading and removal, resulting in formation of different subspecies of lipophorin at different life stages. Lipophorins with different lipid contents have different structures and morphologies.

Phospholipid, another important surface component of all lipoproteins, was studied by ^{31}P-NMR and phospholipase-C (PL-C) treatment. Surface amphipathic apolipoproteins have a significant impact on the mobility of

phospholipid monolayer of lipoprotein particle. The binding of amphipathic lipoproteins is associated with the amount of neutral lipids present, such as diacylglycerol. ^{13}C-NMR study of lipophorins revealed two diacylglycerol pools, representing two different microenvironments, core and surface.

The overall conclusion during the course of study can be described as follows: The integral apolipoprotein frame of both human lipoproteins and insect lipophorins can accommodate a certain amount of hydrophobic lipids in their core. When core space is fully occupied, the neutral lipids will partition to the lipoprotein surface, thereby recruiting amphipathic apolipoproteins to cover those extra hydrophobic areas to stabilize the lipoprotein particles. Lipid transfer particle mediates the movement of lipids among different lipoprotein particles.

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Abbreviations

apo	apolipoprotein
ApoLp	Apolipophorin
BSA	Bovine serum albumin
CD	Circular dichroism
CE	Cholesteryl ester
CETP	Cholesteryl ester transfer protein
DG	Diacylglycerol
DSC	Differential scanning calorimetry
HDL	High density lipoprotein
HDLp-A	High density lipophorin-adult
HDLp-L	High density lipophorin-larva
HDLp-W1	High density lipophorin-wanderer 1
HDLp-W2	High density lipophorin-wanderer 2
HPLC	High pressure liquid chromatogrphy
IDL	Intermediate density lipoprotein
IR	Infred spectroscopy
kDa	Kilodalton
LCAT	Lecithin cholesterol acyltransferase
LDL	Low density lipoprotein
LDLp	Low density lipophorin
LTP	Lipid transfer particle
MTP	Microsomal triacylglycerol transfer protein
NMR	Nuclear magnetic resonance
PBS	Phosphate buffered saline
PC	Phosphatidylcholine

PDI	Protein disulfide isomerase
PE	Phosphatidylethanolamine
PLA_2	Phospholipase A_2
PLC	Phospholipase C
SDS-PAGE	Sodium docecyl sulfate-polyacrylamide gel electrophoresis
TG	Triacylglycerol
TLC	Thin layer chromatography
UV	Ultraviolet spectroscopy
VHDLp-E	Very high density lipophorin- egg
VLDL	Very low density lipoprotein

Chapter 1. Introduction

Hydrophobic compounds are important components in living systems. These materials, which function in a variety of biological roles, are present in all animals. Some of these compounds, such as glycerolipids, provide highly efficient energy sources (Brindley, 1991). Others, like phospholipids and cholesterol, are building materials for biological membranes (Vance, 1991). Sterols are the precursors for different hormones which regulate the metabolic processes in biological systems (Edwards, 1991). The lipophilic vitamins, such as carotene, are essential for life. Some extremely hydrophobic lipids, such as hydrocarbons, are used to protect animals to from harsh environments (Ryan, 1990a). A common characteristic of those compounds is their low water solubility. Transport systems that allow movement of those hydrophobic materials in aqueous media are required for all animals that have a circulatory system. Such transport carriers are often referred to as lipoproteins, which are combined assemblies of lipids and protein(s).

The structures of lipoproteins from human and other vertebrates can be described by a model in which there is a hydrophobic core area occupied by the most hydrophobic compounds, such as triacylglycerol (TG) and/or cholesterol ester (CE). This hydrophobic core is surrounded by a monolayer of amphiphilic lipids and apolipoprotein(s) (Shen *et al.*, 1977; Kézdy, 1978). Packing hydrophobic materials in spherical particles allows efficient solubilization and transport through an aqueous medium, with metabolic fine-tuning accomplished by specific apolipoproteins as well as enzymes and transfer proteins.

1.1 Structure and metabolism of human lipoproteins

Because lipids have a lower buoyant density than water, lipoproteins float when plasma is subject to high-speed centrifugation. Separation of lipoproteins by isopycnic gradient ultracentrifugation produces a distinct banding pattern of the major classes of lipoproteins. The lipoprotein particles with a particular density are classified as follows: chylomicrons, (d<1.00 g/ml), very low density lipoprotein (VLDL) (d<1.006 g/ml), intermediate density lipoprotein (IDL) (d=1.006-1.019 g/ml), low density lipoprotein (LDL) (d=1.019-1.063 g/ml) and high density lipoprotein (HDL) (d=1.063-1.21 g/ml). HDL can be classified further into HDL_2 (d=1.063-1.125 g/ml) and HDL_3 (d=1.125-1.21 g/ml). Chylomicrons and VLDL are specialized for transport of TG while others carry cholesterol and CEs as their major neutral lipids.

The function of chylomicrons is to transport exogenous lipids from their site of absorption (intestine) to sites of utilization (Fielding and Fielding, 1991). The lipid composition of chylomicrons is a reflection of the composition of the diet. For example, a high cholesterol meal will result in relatively small, CE-enriched chylomicrons while a TG-rich meal will result in large, TG-enriched chylomicron particles. Chylomicron particles show a wide spectrum of sizes (diameters from 35 to over 250 nm). VLDL is the lipoprotein particle derived by liver and is responsible for delivering *de novo* (endogenously) synthesized fat (in the form of TG) from liver to peripheral cells.

TG-rich lipoproteins consist of a monolayer surface of phospholipid and free cholesterol which surrounds a hydrophobic core of TG and a small amount of cholesterol ester. The metabolism of TG-rich lipoproteins is rapid and begins almost immediately upon entering blood plasma. TG is rapidly hydrolyzed into free fatty acids by lipoprotein lipase (LPL) which is anchored to endothelial cell surfaces by binding to glycosaminoglycans (e.g. heparin). The lipolysis reaction, however, is facilitated by apolipoprotein C-II, which associates with TG-rich lipoprotein particles after they are secreted into plasma. Patients lacking apo C-II due to genetic deletion, but having normal LPL, display a nearly complete inability to metabolize TG-rich lipoproteins, a defect which can be reversed by complementation with normal human C-II.

Nascent chylomicrons contain not only apo B-48, which plays a role in establishing the structural frame of the particle, but also some apo A-I, the major apoprotein of plasma HDL, and apo E (Fielding and Fielding, 1991). Apo E is greatly increased as soon as the lipoprotein is exposed to plasma. Other apoproteins, specifically the apo Cs, equilibrate from HDL on to the chylomicron surface to activate LPL catalyzed lipolysis. As the lipolysis proceeds the core content of TG decreases and excess surface apolipoproteins dissociate. Without apo C-II the LPL lipolysis is stopped and a chylomicron remnant is formed. The smaller remnant particle will be taken up by the chylomicron remnant receptor (or LDL receptor related protein, LRP) in the liver. Apo E in the remnant particle will be recognized by LRP (Herz *et al.*, 1988).

Newly synthesized VLDL particles found in liver Golgi vesicles have been shown to contain apo E and apo A-I as well as their major structural protein apo B-100 (Hamilton *et al.*, 1991). Both apo E and apo A-I dissociate, and the apo Cs associate augmented from HDL as soon as the particles mix with the plasma. LPL hydrolyzes TGs of secreted VLDL, but less efficiently than from chylomicrons, leading to slower clearance of VLDL compared to chylomicrons. During the prolonged residence of VLDL particles in the circulation, the particles become smaller and their density increases as initially intermediate density lipoproteins (IDL) and finally, low density lipoproteins (LDL) (Schneider, 1991).

1.1.2. LDL structure and metabolism

LDL is catabolized via the LDL receptor which is present in the liver as well as extrahepatic tissues (Goldstein *et al.*, 1985; Brown and Goldstein, 1986; Schneider, 1989). The plasma LDL levels are the results not only of LDL receptor numbers, but also are influenced by the rate of VLDL biosynthesis, the activities of lipoprotein lipase, and possibly by other less well characterized catabolic processes. In addition, receptor-mediated metabolism of hepatically derived lipoproteins is influenced by the direct transfer of CEs from HDL on the intermediates in the conversion of VLDL to LDL, and possibly to LDL particles themselves (Schneider, 1991).

Apo B containing lipoproteins have a common structural organization (Chan, 1992). Either apo B-48 or apo B-100 forms an integral protein frame. Apo B contains a lot of β-sheet structures in which the nonpolar lipids, such as TG and CE, are surrounded by hydrophobic domain of β-sheet. The hydrophilic face of the β-sheet, along with phospholipids and unesterified cholesterol, projects into the aqueous medium. Apolipoproteins associated with lipoprotein particles function not only as structural components but also as a mark directing the lipoprotein particles toward their specific target tissues. For example, both apo B-100 and apo E can be recognized by LDL receptors of cells. Apo E containing chylomicron remnants can interact with LRP (Herz *et al.*, 1988).

1.1.3. Structure and metabolism of HDL

HDL particles contain no apo B apoprotein as its integral protein matrix. The hydrophobic lipids, mainly TG, are wrapped up by phospholipids, unesterified cholesterols and some water soluble surface binding apolipoproteins, like apo A-I, apo A-II, apo C-I, C-II, C-III, and apo E.

Newly synthesized HDL particles contain little CE and are present in a discoidal shape as visualized by electron microscopy. The phospholipids form a 4.5 nm thick bilayer structure with apo A-I probably bound to the equatorial belt of the disc (Hamilton *et al.*, 1976). Excess amounts of free cholesterol on the cell surface are esterified by lecithin cholesterol acyltransferase (LCAT) which is activated by apo A-I (Yokoyama *et al.*, 1980). From its name, we know that LCAT possesses both acyltransferase and phospholipase A_2 activities. The substrates of LCAT reaction are cholesterol and phosphatidylcholine (lecithin), which is hydrolyzed into free fatty acid and lysophosphatidylcholine. Cholesterol is esterified with fatty acid, producing CE. Since lysophosphatidylcholine is water soluble and diffuses away, LCAT reaction can proceed continuously. The newly formed hydrophobic CE molecules pack into the center of the discoidal HDL particles, producing spherical like particles. The CEs of those mature HDL particles are transferred to the IDL and eventually to LDL particles by CE transfer protein (CETP) found in plasma (see section 1.3). LDL particles can be catabolized by liver. This process is called reverse cholesterol transport, in which HDL plays an important role (Hara and Yokoyama, 1991). It is not surprising that HDL particles, although isolated in heterogeneous state, contain no integral structural matrix like apolipoprotein B. The CE core is surrounded by amphipathic molecules, including phospholipids, unesterified cholesterol and surface binding apolipoproteins, mainly apo A-I and apo A-II.

1.2. Lipophorin structure, function and metabolism

The transport of nonpolar materials is not limited to vertebrates. All organisms which have a circulatory system possess some mechanism to solubilize and transport dietary and/or stored lipids to the sites of storage and/or the site of utilization. In invertebrates, such as insects which have an open circulatory system, there are lipoproteins present to solve the water insolubility of lipids. The two best characterized insects regarding to lipid transport biochemistry are the locust, *Locusta migratoria* and the tobacco hornworm, *Maduca sexta*. Insects are the vectors of many communicable diseases. Therefore the study of insect lipoproteins is of great importance for agriculture and medicine. Insect pests destroy crops and therefore have great impact on the economy. Many synthetic hydrophobic pesticides or their metabolites are sequestered and transported by plasma lipoproteins (Haunerland and Bowers, 1985 and 1986). Furthermore, an understanding of insect lipid transport may gain insights into the evolution, structure and function of more complicated mammalian lipoprotein systems.

1.2.1. Structure and composition of lipophorin in *M. sexta*

In order to differentiate from mammalian lipoprotein nomenclature, the term of lipophorin (Lp), coined from the Greek words lipos (lipid) and phoros (bearing) was introduced (Chino *et al.*, 1981) as a convenient generic term for the major lipoprotein in insect plasma. Lipophorin can be easily isolated by density gradient ultracentrifugation based on its buoyant density. Stable lipophorin subspecies that range in density between 1.03 and 1.24 g/ml have been isolated and characterized from *M. sexta*. Those lipophorin subspecies contain different amounts of proteins and lipid, reflecting the different physiological states of the organism with respect to lipid metabolism.

Lipophorin subspecies isolated from different life stages of *M. sexta* are comprised of approximately 40-80 % protein in the form of two nonexchangeable, integral apolipoproteins, although in some cases a third small surface apolipoprotein is present (see below). The major neutral glycerolipid of lipophorin is diacylglycerol (DG) with much lower levels of monoacylglycerol (MG) and TG present in all cases. The DG content of lipophorin varies during development but generally can be considered to be the major neutral lipid component. In mammalian lipoproteins TG is present in sizable amounts as a core component but DG is only present as a minor component.

In mammalian lipoproteins the major amphiphilic lipids are phospholipids, among which phosphatidylcholine (PC) is the dominant species although in some cases, such as human LDL, sphingomyelin is present in a fairly large amount. *M. sexta* lipophorin subspecies contain not only PC but also phosphatidylethanolamine (PE). PC and PE comprise almost all the phospholipid mass.

In mammals, sterols can arise from both dietary and *de novo* synthesis. Cholesterol can be transported by lipoproteins both in free form and esterified with fatty acids within the blood stream. Insects, however, can not synthesize cholesterol *de novo,* which suggests that transport of dietary sterol by lipophorin is an important process. Following administration *in vivo*, nearly all radiolabeled cholesterol in hemolymph is associated with lipophorin (Chino, 1985). Cholesterol serves not only as building material of membranes but also as precursor of the molting hormone, ecdysone, a steroid hormone required for proper development and metamorphosis (Steel *et al.*, 1985). The transport of dietary cholesterol by lipophorin is essential for survival. The major transported sterol by lipophorin is free cholesterol while CE is found in trace amounts.

Long chain straight and methyl branched hydrocarbons are found as surface components of insects and protect againsts dessication of these animals that have a large surface to volume ratio (Blomquist *et al.*, 1987). Lipophorin functions in the transport of hydrocarbons from the site of synthesis (in oenocytes) to the cuticle where they are deposited. Other minor lipid components of lipophorin include carotenes, which have a characteristic yellow color and may be important in gene regulation within the nuclei of cell.

1.2.2. Apolipophorin-I and -II

The SDS-PAGE of lipophorin reveals two apolipoprotein components which are called apolipophorin-I (apoLp-I) and apolipophorin-II (apoLp-II). These two apolipoproteins have similar functions as apo-B in mammalian lipoproteins, which are the constituents of lipoprotein matrix. ApoLp-I is the largest lipophorin apolipoprotein with a molecular weight of about 240,000 daltons and is present in all lipophorin forms. The amino acid compositions for apoLp-I and is similar to mammalian apolipoprotein B (Shapiro *et al.*, 1984; Dillwith *et al.*, 1986; de Kort and Koopmanschap, 1989). A partial amino acid sequence of apoLp-I from locusts has been deduced from the nucleotide sequence of the 2.4 kb cDNA insert from a clone that had been identified immunologically in the locust fat body cDNA library using polyclonal anti-HDLp/antiLp-I antibodies. Comparison of the sequence deduced from this 2.4 kb cDNA to these present in the protein data bank revealed that it has highest score with a fragment of the human apo B-100 precursor. This may indicate an evolutionary relationship between human and insect apolipoproteins (Van der Horst *et al.*, 1993). Circular dichroism spectra of native lipophorin show a structure that is rich in β-sheet (Kashiwazaki and Ikai, 1985) like its mammalian counterpart apo B.

Like apoLp-I, apoLp-II is a constitutive, integral apolipoprotein component of lipophorin particle. The molecular weight of apoLp-II is about 78,000 daltons. Amino acid composition reveals high degree of similarity with apoLp-I. Immunomapping of intact lipophorin by antibodies raised against the individual apolipophorins reveals that apoLp-II is less exposed than apoLp-I (Schulz *et al.*, 1987). Cross-linking studies have shown that apoLp-I and apoLp-II are in close contact as crosslinks are readily formed with dimethylsuberimidate or dimethyladipimidate (Kashiwazaki and Ikai, 1985).

Recent immunoprecipitation experiment showed that apolipophorin I and II originate from a common precursor with a molecular mass of approximately 280 kDa, which was present in the homogenates of *in vitro* labeled fat body of *L. migratoria*. Pulse-chase experiment revealed that this precursor protein is cleaved into apoLp-I and apoLp-II which subsequently are secreted as HDLp from fat body. The time required for complete synthesis and secretion was estimated to be about 35 minutes (Weers *et al.*, 1993)

1.2.3. Apolipophorin-III (apoLp-III)

While apoLp-I and apoLp-II are found associated with all lipophorin forms, apoLp-III associates with only certain lipophorin subspecies, mainly found in the adult moth stage. In resting *M. sexta* adults, for instance, two molecules of apoLp-III are present per lipophorin particle, yielding a high density lipophorin-adult (HDLp-A) particle which has an apoprotein molar ratio 1:1:2 for apoLp-I, apoLp-II and apoLp-III respectively (Kawooya *et al.*, 1984). During flight an adipokinetic hormone (AKH) is secreted into hemolymph. In response to AKH the TG stored in fat body is hydrolyzed into DG by a hormone sensitive lipase and the DG is loaded onto HDLp-A with the help of LTP (see below) to produce low density lipophorin (LDLp). The DG content increases from 25 % in HDLp-A to 46.9 % in LDLp. The diameter of particle increases from 16 nm in HDLp-A to 24 nm in LDLp. Since large amount of DG is loaded onto the particle, it expands. The extra hydrophobic surface area created by the increase of DG needs to be covered. ApoLp-III, present in hemolymph, is brought in to cover the hydrophobic surface. After DG is metabolized into free fatty acids for β-oxidation to provide energy fuel to maintain flight, the particle shrinks back to HDLp-A and apoLp-III is released. HDLp-A and apoLp-III now are ready to reload DG to begin another cycle of lipid utilization.

Recently *L. migratoria* apoLp-III has been crystallized and its three dimensional structure has been solved at 2.5 Å (Breiter *et al.*, 1991). This is the first apolipoprotein whose tertiary structure has been determined. It contains five amphipathic α-helices linked by short loops. In its free state, i.e., not bound to lipids, the five helices form a bundle such that the hydrophobic faces of the helices orient toward each other inward to the bundle and hydrophilic region points toward aqueous medium. It has been proposed that when it binds to lipids the helical bundle opens and the hydrophobic regions of helices, which used to contact each other now cover the hydrophobic lipid surface to prevent the exposure of hydrophobic regions to water. The hydrophilic regions of helices are still projected toward the water. Shortly after the tertiary structure of *L. migratoria* apoLp-III had been reported, the N-terminal fragment of human apolipoprotein E was crystallized and this protein has a very similar three-dimensional structure to that of *L. migratoria* apoLp-III, indicating that these amphipathic surface binding apolipoproteins share a common structure (Wilson *et al.*, 1991).

1.3. Lipid transfer

1.3.1. Mammalian lipid transfer

For a long time it had been generally believed that CEs, because of their water insolubility, could not transfer between lipoprotein particles. The discovery of a plasma CE transfer protein (CETP) (Zilversmit *et al.*, 1975) reversed the old concept, leading to the hypothesis of so called reverse cholesterol transport.

CETP is a single polypeptide glycoprotein with a protein molecular weight of 53,108 Da (Drayna *et al.*, 1987) and a total molecular weight of about 70,000 Da. It catalyses the movement of CE, and to a lesser extent, TG and other non-polar lipids (such as retinyl esters) between plasma lipoproteins. Physiologically, CETP may promote the transfer of LCAT derived CEs out of HDL where they were formed, into VLDL, IDL or LDL, in exchange for TG (Tall, 1986). The removal of VLDL and LDL from the circulation is regulated by the LDL receptor (Schneider, 1989). Although CETP catalyzes the exchange of phospholipids, the rate of transfer would be one magnitude lower than phospholipid exchange protein, which can promote the transfer of the major phospholipids between lipoproteins (Albers *et al.*, 1984).

Microsomal TG transfer protein (MTP) is a soluble protein present in the lumen of microsomes isolated from liver and intestine (Wetterau and Zilversmit, 1986). It mediates the transport of TG, CE, and PC between membranes (Wetterau and Zilversmit, 1985). The ability of MTP to transport TG between membranes, together with its tissue distribution and subcellular location, has led to the suggestion that MTP functions in the assembly of plasma lipoproteins (Wetterau and Zilversmit, 1986). Indeed, it has been shown that a defect in MTP is the basis for abetalipoproteinemia, an autosomal-recessive disease that is characterized by a virtual absence of plasma apo B containing lipoproteins and by low plasma concentration of TG and cholesterol (Wetterau *et al.*, 1992).

MTP is a heterodimer of 58- and 88-kDa peptides (Wetterau *et al.*, 1991a). Characterization of the 58-kDa subunit indicated that it is the previously described multi-functional protein, protein disulfide isomerase (PDI) (Wetterau *et al.*, 1990). The role of PDI in the transfer complex is not known.

At minimum, PDI appears to be necessary to maintain the structural integrity of the transfer protein (Wetterau *et al.*, 1991b), but a larger role cannot be ruled out. Because PDI by itself does not have lipid transfer activity, the 88-kDa subunit is either the active component or it confers transfer activity to the protein complex.

1.3.2. Insect lipid transfer particle (LTP)

Lipophorins isolated from different metabolic stages have different lipid contents and, therefore, different densities. All these subspecies are interconvertible. If two different lipophorin subspecies are mixed together, no significant lipid flux between two lipophorins occurs. However, when a nondialyzable heat labile factor, present in lipophorin deficient hemolymph, was added to the incubation medium there is a dramatic density redistribution of reisolated lipophorins. This led to the discovery of lipid transfer particle (LTP) (Ryan *et al.*, 1986). LTP is also a lipoprotein which contains 14 % lipid mass. It contains three apolipoprotein components, apoLTP-I, -II and -III, which have molecular weights of 320, 85, and 55 kDa, respectively. The molecular weight of intact LTP was estimated about 1,000 kDa based on the native pore limiting PAGE as well as sedimentation equilibrium studies (Ryan *et al.*, 1990d). LTP contains 5% by weight covalently bound sugar (mannose and N-acetyl glucosamine) and each of the three subunits is glycosylated (Ryan *et al.*, 1988a). Since microsomal TG transfer protein (MTP) has similar subunit molecular weights to these of apoLTP-II and apoLTP-III, together with their similar biological function, it has been speculated they might be related.

It has been shown that the 14% lipid in LTP is not merely a structural component of the particle but rather is dynamic and participates in the lipid transfer reaction. Radioactively labeled DG of lipophorin was recovered in the

LTP lipid moiety when LTP was incubated with lipophorin, and *vice versa*, radioactively labeled lipid in LTP could be transferred back to unlabeled lipophorin. When lipid of LTP was removed by either organic solvent extraction or by detergent treatment, transfer activity is lost. Electron microscopy of LTP reveals an asymmetric shape which has a spherical head region and an elongated cylindrical tail linked by a central hinge. After treating LTP with SDS, the head region was perturbed suggesting that lipids might locate in this head region (Ryan *et al.*, 1990b). Polyclonal antibodies raised against purified LTP in rabbits demonstrated that lipid transfer activity could be blocked if antiserum was added to the incubation medium (Ryan *et al.*, 1988b).

1.3.3. LTP facilitated lipid transfer

When different lipophorin subspecies which contain different amounts of DG were incubated together in the presence of LTP, a new equilibrium was established in which a lipophorin subspecies of a density between that of starting lipophorins was produced. Thus it was concluded that the observed changes were due to facilitated redistribution of lipophorin-associated lipid. The facilitated vectorial net transfer of lipid mass from lipid-rich donor lipoprotein to relatively lipid-poor acceptor lipoprotein redistributed the lipid complement of the original substrate particles such that, ultimately, each particle possessed similar amounts of lipid while retaining its original apoLp-I and apoLp-II complement. There was no observable migration of apolipophorin subunits among different starting lipophorins. This supports the concept that apoLp-I and apoLp-II are integral, nonexchangeable matrix of lipophorin particles. Lipophorin could be used as a shuttle vehicle to transport lipids among different subspecies. LTP mediates lipid loading onto the lipophorin acceptor from a lipid donor, and/or the depositing lipids from lipophorin donor to a lipid acceptor.

Lipid transfer between lipid donor and acceptor facilitated by *M. sexta* hemolymph LTP is not restricted to *M. sexta* lipophorin subspecies. It has been shown that a variety of lipids could be transferred by LTP as well as different lipoproteins including an unrelated insect plasma very high density chromolipoprotein (Haunerland and Bowers, 1985b; Ryan *et al.*, 1988a), human LDL and a human apolipoprotein A-I stabilized phospholipid/triolein microemulsion (Ando *et al.*, 1990). The latter two substrates have been employed as donor/acceptor species in experiments which illustrate the difference in catalytic activity of LTP versus human plasma CE transfer protein (CETP) (Tall, 1986). While CETP promotes hetero-exchange of TG and CE between these pools (Morton and Zilversmit, 1982, Nishikawa *et al.*, 1988), LTP catalyzed net transfer of TG and phospholipid mass from emulsion to LDL produces a lipid-enriched LDL that retains its original apolipoprotein profile. Other studies have shown that lipophorin can serve as a lipid donor upon incubation with LDL and LTP. Facilitated vectorial transfer of DG from lipophorin to LDL produces a DG-enriched LDL of lower density and increased lipid:protein ratio and a lipid-depleted lipophorin whose properties are similar to egg very high density lipophorin VHDLp-E (see chapter 2; Ryan *et al.*, 1990c; Kawooya *et al.*, 1988).

1.3.4. LTP induced human high density lipoprotein (HDL) transformation reaction (Silver *et al.*, 1990)

When catalytic amount of LTP incubated with human high density lipoprotein (HDL) at 37 °C, the lipid and protein profiles were totally different from that of starting HDL after centrifugation. One population has a very low density containing very high lipid content, mainly CE originally from HDL, plus phospholipids and apolipoprotein. Unlike starting HDL the major apolipoprotein in this less dense population are apolipoprotein A-II and a small amount of apolipoprotein A-I while starting HDL contains a much higher content of apolipoprotein A-I. Another population lacking lipids is mainly apolipoprotein A-I which is recovered at the bottom of the tube. The radii of lipoprotein particles increased from 4-5 nm in HDL (HDL_2 and HDL_3) to 9-10 nm in transformed products. Since LTP has the ability to facilitate lipid transfer among the different lipoprotein particles, it is reasonable to think that it could also transfer the lipids from one HDL particle to the other to create a larger, less dense particle in which the ratio of surface to volume was greatly reduced. The newly generated particle has less hydrophobic area which has to be covered by surface apolipoproteins. Having a higher lipid binding affinity than apolipoprotein A-I (Lagocki and Scanu,1980; Rosseneu *et al.*, 1981; Edelstein *et al.*, 1982), apoA-II molecules remain associated with the newly transformed product while the excess apoA-I molecules, originally from starting HDL particles, dissociate from the surface of lipoprotein particle. It has been shown that a similar transformation reaction took place by heating HDL solution to 60-70 °C (Tall *et al.*, 1977). Between 50-60 °C temperature range it had been observed that apolipoprotein A-I was released from HDL particles. This human HDL transformation product is thermodynamically more stable than HDL. The transformation reaction could be carried out either at high temperature without LTP or at low temperature in the presence of LTP. Catalytic amount of LTP decreases the energy barrier of the transformation and therefore the reaction takes place at lower temperature, generating a more stable transformed product.

1.4. Rationale of lipoprotein structural studies

1.4.1. Morphologies and structural organization of apolipoprotein matrix

1..4.1.1. Lipoprotein interconvertibility among different subspecies

Insect lipophorins isolated from different life stages have different compositions which reflect the dynamic metabolic states at a particular life stage. A common structural component of these lipoprotein particles is they all contain an integral protein frame comprised of apoLp-I and -I, from which lipid, mainly DG, can be deposited or removed. Different amounts of DG associated with apolipoprotein, together with phospholipids, would result in different morphologies which can be visualized by electron microscopy. Different amount of neutral lipid could also affect the accessibility of apolipoprotein frame to the proteolysis and/or other biochemical probe(s).

1.4.1.2. *In vitro* retailoring lipoprotein particles via LTP mediated lipid transfer

There is increasing evidence that LTP catalyzed lipid transfer is responsible for the lipophorin subspecies interconversion *in vivo*. When human LDL is incubated with insect lipophorin together with LTP, the lipid composition would be totally changed. DG originally associated with lipophorin has been recovered in human LDL fraction. This lipid transfer reaction permits us to create two new lipoproteins, a lipid devoid lipophorin and a DG enriched LDL particle. Studies of these newly formed lipoproteins would provide some insight into their structures. Human HDL particles have a different structural feature. They do not contain an integral protein matrix. The oily lipid core is surrounded by phospholipids and amphipathic exchnageable apolipoproteins. In the presence of LTP, HDL particles transform into a LDL sized particle containing no apo B. This transformation product is an excellent substrate to study for the comparison with native LDL particle by many biochemical and biophysical tools. Furthermore since apo A-I dissociates from the particle because of the decreased surface areas in the transformed product, this reaction provides a convenient means to purify apo A-I without denaturation.

1.4.1.3. LDLp formation and metabolism *in vivo*, binding and dissociation of amphipathic apolipoprotein

During flight, adipokinetic hormone (AKH) stimulated fat mobilization in the fat body of adult moths functions to meet an increased demand for energy fuel. TG molecules stored in fat body are hydrolyzed into DG molecules. Newly formed DG molecules are loaded onto pre-existing circulating lipophorin, HDLp-A, thereby forming low density lipophorin (LDLp). As discussed earlier, increased amount of DG results in the increased particle size and decreased density. The amount of phospholipid does not increase correspondingly. Fourteen additional molecules of apoLp-III, present in the hemolymph, associate with LDLp. When fuel molecules, DG, are metabolized for energy production, the LDLp particle is converted back to HDLp-A which is the substrate for the next round of lipid loading. During this process, 14 apoLp-III molecules of LDLp dissociate from the particle as a result of decreasing DG content.

Since the binding of amphipathic apolipoprotein to lipoprotein particles is a function of the amount of hydrophobic lipid surface, different approaches can be used to establish such a relationship. Firstly, LTP is an excellent tool to study the effect of facilitated lipid transfer on the structures of both donor lipophorin and acceptor LDL. Secondly, we would like to study the structural integrity of lipoproteins after enzymatic conversion of amphipathic phospholipids to hydrophobic lipids (see below).

1.4.2. Phospholipid monolayer of lipoprotein particles

1.4.2.1. Mobility of phospholipid monolayer, a ^{31}P-NMR study

The linewidth of nuclear magnetic resonance is a combined reflection of both particle tumbling in the medium and intrinsic lateral diffusion of the individual component. The manipulation of particle tumbling could be achieved by changing the medium viscosity and/or temperature. We chose four different lipophorin subspecies which contain different amounts of DG molecules. The spectra of individual lipophorins at different temperatures were measured. From the linewidth of phospholipids, the mobility of phospholipid monolayer could be deduced. Such mobility reflects the interaction between phospholipids and apolipoproteins, as well as the core lipids.

1.4.2.2. Effect of enzymatic conversion of phospholipid to diacylglycerol by phospholipase-C

Phospholipids play an important role of stabilizing lipoprotein structural integrity. In human circulation, the phospholipases would attack the lipoprotein particles. It has been suggested that the hydrolytic removal of polar phosphocholine head group of human LDL by either sphingomyelinase or phospholipase-C results in the formation of atherogenic LDL aggregates (Suits *et al.*, 1988; Xu and Tabas, 1991; Steinbrecher and Lougheed, 1992). The aggregation process is the result of transformation of amphipathic phospholipids to much more hydrophobic lipids on the lipoprotein particle surface. We are interested in the possibility of the prevention of PL-C induced LDL aggregation by amphipathic apolipoproteins (Singh *et al.*, 1992). Since these apolipoproteins normally associate with hydrophobic lipids, this may be a mechanism to repair surface lesions on lipoprotein particles caused by these lipases.

1.4.2.3. Correlation between amphipathic apolipoprotein binding and the amount of hydrophobic lipids on the surface of lipoproteins

Unlike human lipoproteins which contain CE and TG as their major core lipids, DG is the major core lipid in insect lipophorins. Apolipophorin-I and -II provide a flexible vessel to deal accommodate different DG contents during different life stages for their metabolic requirements. The hydrolytic product of phospholipase-C (PL-C) is DG. PL-C treated lipophorins will generate DG *in situ* at the expense of surface phospholipids. It is interesting to examine how the apoprotein frame changes to deal with these newly formed DG. We could expect that the apoprotein matrix could accept a certain extent of DG, but would require more amphipathic surface components if newly generated DG exceeds its core capacity.

1.4.2.4. Distinguishing surface and core DG pools by ^{13}C-NMR

DG is an important lipid in the signal transduction pathway in mammalian cells. It is the major energy source for insect migration. During flight, large quantity of DG molecules derived by hydrolysis of TG from fat body are loaded into HDLp-A, forming LDLp. In addition to DG, LDLp contains more surface amphipathic apoLp-III molecules, suggesting that some DG molecules partition to the surface of lipoprotein particle and induce the association of apoLp-III. Conversion of phospholipids to DG in lipophorins by phospholipase-C provides an excellent tool to form surface DG that is different from intrinsic DG molecules. Because of different microenvironments, natural abundance carbonyl ^{13}C-NMR resonances of DG should be different between core and surface pools. By this measurement we can obtain direct evidence of the presence of two distinct pools of DG.

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Chapter 2

Role of lipid transfer particle in transformation of lipophorin in insect oocytes

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Introduction

The lipid movement among the different lipoprotein particles is the major topic in this Chapter. In oviparous animals oocyte development depends on the uptake of lipid and protein constituents from the plasma compartment. In insects, vitellogenin (1), the high density lipoprotein, lipophorin (2) as well as other plasma proteins (3) are taken up by, and accumulate in, developing oocytes. While it is generally recognized that proteolytic degradation of vitellin and lipoproteins occurs during embryonic development in both vertebrates and insects, differences exist between these animals in early postendocytotic processing of these materials. In avian and amphibian species specific postendocytotic apoprotein proteolysis of vitellogenin (4,5), very low density lipoprotein (6) and riboflavin binding protein (7) occurs prior to deposition in yolk granules. In insects, however, uptake and deposition of vitellogenin (3), lipophorin (3,8,9) or microvitellogenin (3) is not accompanied by proteolysis. Minor differences between vitellogenin and vitellin have been demonstrated which appear to result solely from loss of lipid from the particles (10-12). In a similar manner, upon uptake, lipid is removed from high density lipophorin-adult (HDLp-A, density = 1.078 g/ml; 13) which is transformed into egg very high density lipophorin (VHDLp-E; density = 1.24 g/ml; 8,9). Thus VHDLp-E has a smaller size, higher density and lower lipid content compared to its precursor, HDLp-A. Despite the significant loss of lipid, VHDLp-E retains its particle structure as well as its two high molecular weight, integral apoproteins, apolipophorin I (apoLp-I; M_r = 240,000) and apolipophorin II (apoLp-II; M_r = 80,000, 9). Two molecules of apolipophorin III (apoLp-III; M_r = 18,000) originally present on HDLp-A (13-15), however, dissociate from the particle during transformation (9). While the precursor-product relationship between HDLp-A and VHDLp-E is well established, the mechanism whereby this transformation occurs, or its potential physiological significance, is unknown.

M. sexta hemolymph possesses a lipid transfer particle (LTP) capable of catalyzing exchange and net transfer of diacylglycerol (DG) and other lipids between isolated lipophorins *in vitro* (16-19) as well as between lipophorin and human low density lipoprotein (LDL; 20). In these cases LTP-induced redistribution of the lipid content and composition of lipophorin particles occurs without affecting their content of apoLp-I and apoLp-II. Based on the directional flux of LTP-mediated lipid transfer between lipoprotein substrates in transfer assays *in vitro*, we hypothesized that LTP may be involved in formation of VHDLp-E by facilitating the removal of lipid from HDLp-A within the oocyte. The potential implications of this process with respect to receptor-ligand interactions are discussed.

Material and Methods

Animals. *Manduca sexta* were reared on a high wheat germ diet as described elsewhere (21). Eggs were harvested from adult female moths two days after eclosion according to Kawooya *et al.* (9).

Lipoprotein and LTP isolation. Human LDL (density 1.006-1.063 g/ml) was isolated from fasting human plasma by sequential density gradient ultracentrifugation. HDLp-A was isolated from adult male moths on the first or second day following eclosion (19). For some experiments HDLp-A was iodinated with $Na^{125}I$ (Amersham, 100 Ci/ml) as described elsewhere (19,22).

VHDLp-E was isolated from freshly harvested or frozen (-70°C) *M. sexta* eggs according to the density gradient ultracentrifugation procedure described by Kawooya *et al.*. (9). LTP was isolated from hemolymph obtained from prepupal animals seven days following the molt to the fifth larval instar according to Ryan *et al.* (23). LTP was isolated from *M. sexta* eggs by a modification of procedures established for hemolymph LTP as follows. Oocytes were harvested from about 100-150 2-5 day old adult female moths, dissected free of fat body and connective tissue, and thoroughly rinsed with phosphate-buffered saline (PBS; 0.10 M sodium phosphate, pH 7.0, 0.15 M NaCl, 5 mM EDTA). The eggs were then homogenized in PBS containing 5 mM glutathione and the following protease inhibitors: 0.02 M diisopropylphosphoro-fluoridate; 3 mM pepstatin A; 2 mM aprotinin; 1 mM phenylmethanesulfonyl fluoride; 2 mM antipain and 1 mM leupeptin. The homogenate was centrifuged (17,000 x g, 15 min at 4° C) to remove debris. The supernatent was collected and brought to a density of 1.31 g/ml by the addition of solid KBr (44.5 % w/v) in a final volume of 20 ml. This solution was transferred to a Beckman Quick-Seal tube and overlayered with 20 ml of a 33.4% KBr (w/v) solution in PBS. The sample was then subjected to ultracentrifugation at 50,000 rpm for 16 h at 4 °C in a Beckman VTi 50 rotor. After centrifugation the top 8 ml of solution was collected and dialysed against 50 mM sodium phosphate buffer (pH 7.5). The sample was then applied to a column (2.5 x 20 cm) of DEAE Bio-Gel A (Bio-Rad) pre-equilibriated with 50 mM sodium phosphate buffer (pH 7.5) and washed with the same buffer to remove non adsorbed protein. Application of a linear gradient of salt (0.0-0.4 M NaCl in 50 mM sodium phosphate buffer, pH 7.5) resulted in elution of LTP. The fractions corresponding to LTP were pooled, dialyzed against 50 mM phosphate pH 7.5 and applied to a mini column (1.0 x 3.0 cm) of DEAE Bio-Gel A. Bound protein was then eluted from this column with buffer containing 0.4 M NaCl. The latter step provided a convenient method to concentrate the sample.

Production of anti-LTP serum and isolation of anti-LTP IgG.

Polycolonal Anti-LTP serum was raised in a young adult New Zealand white rabbit as described elsewhere (18) and the IgG fraction was purified from collected serum by a combination of ammonium sulfate precipitation (33 % saturation) and chromatography on a column of DEAE Affi-Gel Blue (Bio-Rad) according to the manufacturer's instruction.

Lipid Transfer Assay. Routine quantitative assay of LTP activity was performed using human LDL and insect ^{3}H-DG labeled high density lipophorin-larval [18] as donor/acceptor according to Ryan *et al.* (23). Alternatively HDLp-A (1 mg protein) was incubated with 2.2 mg human LDL protein for 1 h in the presence or absence of 10 µg insect LTP at 33° C. Following incubation the lipoprotein substrates were reisolated by density gradient ultracentrifugation. The sample was brought to 33 % KBr (w/v) in a volume of 20 ml, transferred to a 39 ml Beckman Quick-Seal tube, overlayered with 0.9 % saline and centrifuged at 50,000 rpm for 4 h at 4° C in a VTi 50 rotor . Following centrifugation the tube contents were fractionated from the top and the density, protein, lipid or radioactivity in each fraction were determined. A standard curve constructed from the transfer activity of known amounts of purified LTP was used to determine the LTP concentration in the buffer soluble fraction of oocyte homogenates.

***In vitro* incubations of egg homogenates.** Approximately one hundred freshly harvested oocytes (0.5 - 0.8 mm diameter) were homogenized in 1.5 ml lepidopteran saline (5 mM K_2HPO_4, pH 6.5, 110 mM KCl, 4 mM NaCl, 15 mM $MgCl_2$, 4 mM $CaCl_2$). Aliquots (32 mg oocyte protein) of the homogenate were then used for *in vitro* transformation experiments. Incubations were carried out under argon atmosphere to minimize melanization of the sample.

Analytical Procedures. SDS-PAGE was performed according to Laemmli (24) on 4-12 % acrylamide gradient slab gels run for 3.5 h at 30 mA. Protein was assayed with the BCA assay (Pierce Chemical Co.) using bovine serum albumin as a standard. Lipid analyses were performed using enzymatic kits obtained from Wako Pure Chemicals. Total and free cholesterol, choline containing phospholipids and neutral acylglycerols were assayed in individual lipoprotein containing fractions obtained following density gradient ultracentrifugation. Density determinations of fractions obtained following density gradient ultracentrifugation were made by refractometry. Radioactivity was determined by liquid scintillation counting for tritium containing samples and with a gamma counter (Beckman model 5500) for ^{125}I containing samples. Double radial immunodiffusion was performed according to Ouchterlony (25).

Results

LTP-catalyzed conversion of HDLp-A into a VHDLp *in vitro*.

Incubation of insect HDLp-A with human LDL in the presence of catalytic amounts of LTP results in significant alteration in lipoprotein density distribution (Figure 2.1). In control incubations without LTP no changes in lipoprotein density were observed. LDL and HDLp-A were well separated and had floatation properties identical to those obtained when centrifuged separately. In the presence of LTP, however, LDL shifted to a slightly lower density while HDLp-A was replaced by a new lipoprotein peak at the bottom of the density gradient. This new material migrated to a position corresponding to a VHDLp (density = 1.21-1.28 g/ml). This reaction, therefore, is similar to that previously observed with larval high density lipophorin (20) indicating lipophorins possessing quite distinct physical properties (27) react in a similar manner and form stable end products of the same density.

The apoprotein content of lipoprotein populations obtained from control and LTP containing incubations was then analyzed by SDS-PAGE (Figure 2.2). The results show that there was no significant exchange or transfer of apolipoprotein among the donor/ acceptor lipoprotein populations indicating that the observed alterations in lipoprotein density distribution are the result of facilitated lipid transfer rather than LDL-lipophorin particle fusion. From preliminary analysis of lipophorin-containing density fractions we were unable to determine if the VHDLp product lipoprotein retained the two apoLp-III molecules originally present on HDLp-A (13). Thus HDLp-A from the control incubation and the VHDLp product from an LTP-containing incubation were chromatographed on a column of Sephadex G-75 (13) to separate free apoLp-

III from that bound to lipophorin. The electrophoretic pattern obtained (Figure 2.2) shows that both HDLp-A (lane 2) and the product VHDLp (lane 1) contain apoLp-I and apoLp-II but the VHDLp lacks apoLp-III (M_r = 18,000). Thus, in a manner analogous to the transformation of HDLp-A to VHDLp-E *in vivo*, this LTP-mediated transformation of HDLp-A to a VHDLp results in dissociation of apoLp-III. Interestingly apoLp-III dissociation from HDLp-A as a result of lipid transfer was not accompanied by an association with LDL. This observation likely stems from the relatively larger amount of LDL in the incubations, its lipid binding capacity and the known stability of apoLp-III in solution. In other studies (Singh and Ryan, unpublished results) when the amount of HDLp-A was increased relative to LDL, apoLp-III association with LDL was observed as a function of lipid transfer.

Lipid analysis of the VHDLp product lipoprotein was performed and compared with the known compositions of HDLp-A (19) and VHDLp-E (9) (Table I). For both VHDLp produced by lipid transfer *in vitro* and VHDLp-E isolated from oocytes, protein (in the form of apoLp-I and apoLp-II) accounts for the bulk of the particle mass. This contrasts with the protein content of HDLp-A and indicates that LTP has induced net transfer of lipid mass originally present on HDLp-A to acceptor LDL particles. The lipid composition of VHDLp-E isolated from oocyte (9) is similar to that of the VHDLp produced by incubation of LDL and HDLp-A in the presence of LTP. The relatively higher content of cholesterol in VHDLp fraction is likely due to transfer of LDL-associated cholesterol to lipophorin during incubation. From the observed differences in lipid composition between VHDLp and its precursor in this *in vitro* lipid transfer reaction, HDLp-A, it is clear that in the presence of a suitable lipid acceptor (e.g. LDL) LTP has the capacity to catalyze vectorial transfer of a significant portion of HDLp-A associated lipid, thereby transforming it into a product particle with properties similar to VHDLp-E.

Isolation of LTP from M. sexta oocytes. The ability of LTP to catalyze transformation of HDLp-A to a VHDLp suggested that facilitated lipid transfer may be responsible for production of VHDLp-E from HDLp-A in *M. sexta* oocytes. Since the transformation is known to occur within the oocyte we designed experiments to determine if oocyte homogenates possess transfer activity and, if so, attempt to characterize the factor(s) responsible. Initially we conducted transfer assays with crude oocyte homogenates and found transfer activity present. To exclude the possibility that the observed activity was due to hemolymph LTP that was associated with the exterior of the oocytes despite repeated washings, oocytes were exposed to trypsin and subsequently, soybean trypsin inhibitor, prior to homogenization. Trypsin treatment of intact oocytes, under conditions known to significantly reduce LTP activity (23), had no effect on the transfer activity of oocyte homogenates indicating the transfer activity resides within the oocyte.

We then set out to determine whether this activity had properties similar to plasma LTP or was perhaps an as yet uncharacterized lipid transfer catalyst. Initially an oocyte homogenate was subjected to density gradient ultracentrifugation, fractionated and assayed for transfer activity. Transfer activity was recovered only in fractions of density between 1.21 and 1.25 g/ml suggesting the active principle may contain lipid. Based on the possibility that a transfer catalyst with properties similar to plasma LTP (density = 1.23 g/ml; 18) is present in oocytes and may be responsible for the observed activity, we employed a purification scheme similar to that used to isolate LTP from *M. sexta* hemolymph. Through all steps of the purification the properties of the oocyte transfer activity were indistinguishable from that of plasma LTP. Analysis of the active fraction by SDS-PAGE (Figure 2.3) revealed the presence of the three apoprotein components characteristic of hemolymph LTP (apoLTP-I, M_r ~ 320,000; apoLTP-II, $M_r = 85,000$; apoLTP-III, $M_r = 55,000$; 18). To characterize the relationship between the material isolated from oocyte homogenates and hemolymph LTP, double radial immunodiffusion versus anti-LTP serum raised against purified hemolymph LTP, was performed (Figure 2.4). A single fused precipitin line was observed between oocyte-derived and hemolymph LTP indicating identity. From transfer activity measurements performed on oocyte homogenates we estimate the concentration of LTP to be 0.2 ± 0.1 µg LTP/ mg buffer soluble oocyte protein (n = 12). The intraoocytic location of LTP as well as its source are not yet known. It is conceivable that LTP could arise from *de novo* synthesis by follicle cells or, alternatively, via uptake from hemolymph.

Anti-LTP IgG inhibits transformation of HDLp-A to VHDLp-E *in vitro*. Previously we have demonstrated that anti-LTP serum can inhibit the catalytic activity of LTP (18). Thus we set out to determine if anti-LTP IgG was capable of blocking the conversion of HDLp-A to VHDLp-E by oocytes *in vitro*. Preliminary experiments with isolated oocytes were inconclusive perhaps due to an inability of sufficient antibody to be internalized by the oocyte. Thus we turned to the use of oocyte homogenates and found that upon incubation of ^{125}I-HDLp-A with an oocyte homogenate a major change in the density distribution of labeled HDLp-A occurred compared to control HDLp-A incubated in buffer (Fig. 2.5). The observed shift was to a density corresponding to that of VHDLp-E and indicates that all the necessary components for this transformation are retained in oocyte homogenates. Although pre-immune serum had no effect, when anti-LTP IgG was included in oocyte incubations together with ^{125}I-HDLp-A the transformation was inhibited to a large extent and the bulk of the ^{125}I-HDLp-A was recovered at a density position slightly greater than that of the control. When exogenous isolated hemolymph LTP was added to this incubation, however, the inhibition was relieved and the radiolabeled lipophorin was recovered at a density corresponding to that of VHDLp.

Discussion

Oocyte devolopment in insects involves the deposition of large amounts of lipid. In most cases this lipid is primarily derived from plasma lipoproteins rather than synthesis by oocyte tissue *de novo*. In *M. sexta* oocyte lipid stores are predominantly derived from hemolymph lipophorin via one of two pathways which ultimately account for up to 95 % of the oocyte lipid stores with vitellogenin contributing the bulk of the remaining 5 % (26). Kawooya *et al.* (9) showed that lipid can be delivered to oocytes from low density lipophorin (LDLp; 27) by a nonendocytotic mechanism. Alternatively, the major lipoprotein present in resting adult hemolymph, HDLp-A, is taken up via an apparent receptor mediated endocytosis and transformed into VHDLp-E through specific loss of lipid and apoLp-III dissociation. VHDLp-E is a stable lipoprotein particle that has been isolated and characterized (8,9). An important feature of the transformation of HDLp-A to VHDLp-E in *M. sexta* is that postendocytotic proteolytic processing is not involved in this transformation. Indeed, the reaction appears to be restricted to lipid removal with concomitant dissociation of apoLp-III from lipid-poor product particles. The lack of lipophorin proteolysis is similar to the situation with insect vitellogenins but contrasts sharply with the specific postendocytotic processing of vertebrate vitellogenins (4) and lipoproteins (6).

In *Manduca sexta* hemolymph lipophorin exists in a multitude of forms that differ in lipid content and composition but have similar apoprotein components. Different forms of lipophorin that appear during development have been shown to arise from a remodeling of pre-existing particles (21). Thus it has been proposed that nascent lipophorin particles secreted by fat body cells mature to form circulating high density lipophorin particles by uptake of DG from midgut (28). Similarly HDLp-A may beconverted to LDLp by uptake of the DG and additional 14 molecules of apoLp-III (14, 29-31). This latter conversion has been shown to be mediated by hemolymph LTP, which catalyzes transfer of DG from the fat body membrane to HDLp-A, in response to hormonal stimulation (32). The observed interconvertibility of lipophorin subspecies has led to the hypothesis that all lipophorins share the same basic matrix structure (33) which comprises the integral apolipoproteins, apoLp-I and apoLp-II and plus a small amount of phospholipid. Thus addition or depletion of lipid from lipophorin particles does not destroy its basic matrix structure, thereby permitting the particle to function as a reusable lipid shuttle (2). The lipophorin conversion whereby hemolymph-derived HDLp-A is transformed into VHDLp-E within the oocyte can be considered analogous to lipophorin conversions which occur within the plasma compartment. This transformation involves removal of significant quantities of lipid from the particle and therefore could conceivably be catalyzed by either a lipolytic enzyme or a lipid transfer catalyst. Lipolysis, however, cannot explain the specific removal of HDLp-A associated hydrocarbon, carotenes or sterols (9). By contrast, LTP has been shown to be relatively nonspecific with respect to lipid substrate specificity, transferring a variety of lipid classes associated with lipophorin (34).

In an earlier study of its lipoprotein substrate specificity, hemolymph LTP was demonstrated to catalyze vectorial net transfer of DG from lipophorin to LDL producing a DG enriched LDL and a DG depleted lipophorin (20). In the present study it was observed that upon incubation with human LDL and LTP, HDLp-A undergoes a transformation reminiscent of that which occurs upon its uptake by oocytes *in vivo.* In this reaction LDL serves as a lipid sink which, in the presence of LTP, accepts lipophorin-associated lipid, thereby producing the lipid-poor VHDLp. Thus it is conceivable that the function of LDL in this *in vitro* reaction is similar to that of intraoocytic lipid droplets whereby both serve as acceptors of HDLp-A-derived lipid during its conversion to a VHDLp. Based on this observation we hypothesized that a lipid transfer factor may be responsible for transformation of HDLp-A to VHDLp-E *in vivo.* In preliminary experiments we obtained evidence that egg homogenates contain lipid transfer activity and, using a modified purification scheme designed for isolation of hemolymph LTP (23), a protein fraction with properties that are immunologically and electrophoretically indistingiushable from hemolymph LTP was isolated from *M. sexta* egg homogenates. This finding represents the first demonstration of LTP in a tissue other than hemolymph and supports the concept that LTP present in the oocyte mediates transformation of HDLp-A to VHDLp-E. Further evidence for this postulated role was obtained from *in vitro* experiments in which anti-LTP IgG inhibited transformation of ^{125}I-HDLp-A to a density corresponding to VHDLp-E. This inhibitory effect could be overcome, however, by addition of exogeneous LTP.

Functions which have been ascribed to specific postendocytotic proteteolytic processing of vertebrate yolk protein precursors, vitellogenin and very low density lipoprotein, include intraoocytic routing of yolk protein, a regulatory step in fusion between endocytic compartments as well as a potential role in mediating receptor-ligand interaction (5,6). The lack of such processing in insects raises the possibility that alternative control mechanisms may exist for these events. Previous work has shown that the only detectable changes in insect vitellogenin and lipophorin upon uptake by oocytes result from alteration of the lipid moiety of the respective particles. Taken together with evidence presented here of lipid transfer activity within the oocyte, we postulate that facilitated lipid transfer may play an important role in oocyte development through its mediation of lipid redistribution. Tsuchida and Wells (35) have recently shown that lipophorin receptors on fat body membranes have an increased affinity for lipid-rich particles and have proposed this as a possible basis for receptor-ligand interaction. No information is available, however, on the mechanism whereby lipophorin-associated lipids are specifically removed at this tissue. In insect oocytes the presence of specific vitellogenin receptors has been demonstrated (reviewed in 3) and accumulating evidence[1] (3) suggests that lipophorin is also sequestered via receptor-mediated endocytosis (9). Thus it is possible to speculate that, in the case of lipophorin and perhaps vitellogenin, receptor-ligand binding may be affected by the lipid content of the particles and LTP-mediated changes in the lipid content of these ligands may induce or accelerate ligand dissociation, thereby permitting transit to and accumulation within yolk granules as well as receptor recycling. Clearly further work is required to determine the effect of modulation of particle lipid content on its metabolic fate but the potential involvement of a lipid transfer catalyst raises the possibility that novel mechanisms of processing of yolk protein precursors may be operative in invertebrate species. Furthermore, as mentioned above, a second source of lipid for the developing oocyte involves a nonendocytotic mechanism for selective uptake of LDLp-associated DG (9). It is possible that oocyte LTP functions in this process in a manner analogous to its role in lipid loading of lipophorin at the fat body (32) and lipid removal at the flight muscle (33).

Another important observation should bear in mind is that, during the transformation from HDLp-A to VHDLp-E, apoLp-III originally bound to HDLp-A is dissociated from the particle after lipid is removed. This reflects the biological role of apoLp-III, which is a amphipathic apolipoprotein in response to the lipid binding requirements in lipophorin metabolism. In Chapter 4 we will examine the relative lipid binding affinity of apoLp-III. In Chapter 6 we will study the effect of the binding of apoLp-III on the monolayer movement of phospholipids in lipophorin particles. In Chapters 7 and 8 we will describe how the artificially lipid-enriched lipoprotein particle promote the binding of apoLp-III.

[1] H. Liu and R.O. Ryan, unpublished results

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Table 2.1 Composition of lipophorins

	Weight %		
	HDLp-A[a]	VHDLp-E[b]	VHDLp
Phospholipids	14.0	14.4	14.7±1.6
Glycerolipids	27.5	4.6	4.7±0.1
Total cholesterol	1.3	0.7	5.1±0.4
Other lipids	8.7	0.3	N/D
Protein	48.5	80.0	75.5±1.4

[a] Ryan *et al.* (1986). *J. Biol. Chem.* **261**, 563-568

[b] Kawooya *et al.* (1988). *J. Biol. Chem.* **263**, 8740-8747

Figure Legends

Figure 2.1. Effect of LTP on lipoprotein density distribution.

Human LDL (2.2 mg protein) was incubated with HDLp-A (1 mg protein) in the presence (*closed circles*) and absence (open *circles*) of 10 µg LTP for 1 h at 33 °C. Following incubation, the samples were adjusted to a density of 1.23 g/ml by addition of solid KBr (20 ml final volume), transferred to a 39.5 ml Quick-Seal tube, overlayered with 0.9 % saline and centrifuged (50,000 rpm in a Beckman VTi50 rotor for 4h at 4 °C). Following centrifugation the tube contents were fractionated from the top into 1.1 ml fractions. The density and protein in each fraction were determined.

Fig. 2.1

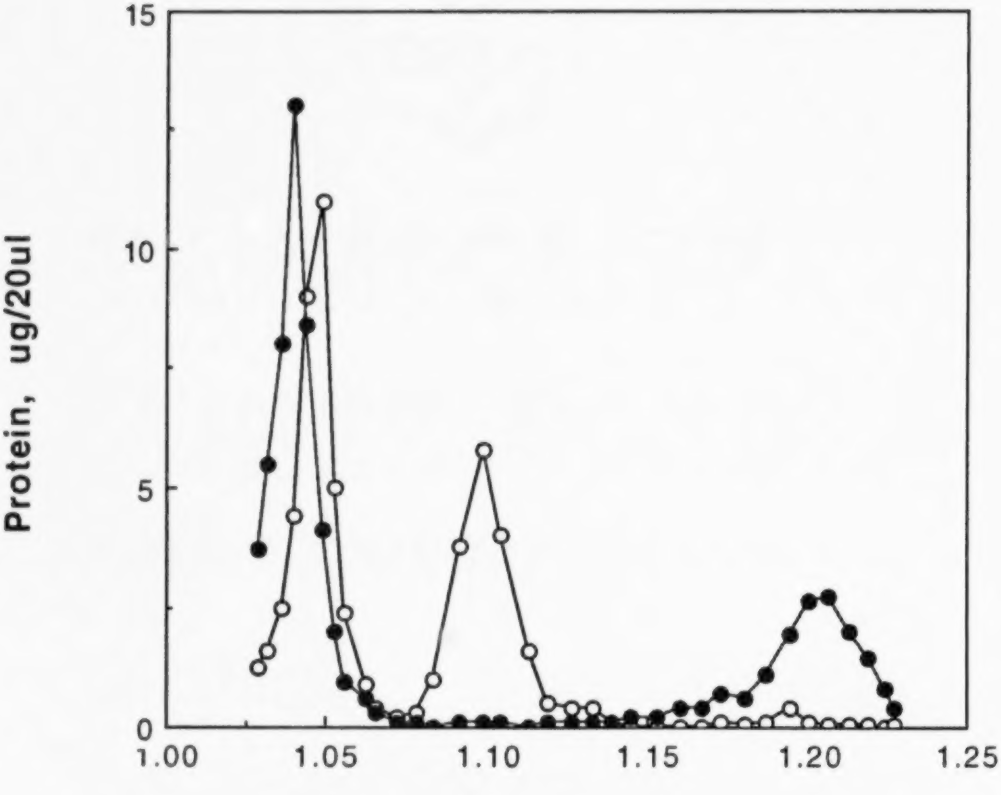


Figure 2.2. SDS-PAGE of lipoprotein fractions. Human LDL and HDLp-A were incubated in the presence or absence of LTP as described in the legend to Fig. 1. Following incubation the samples were subjected to density gradient ultracentrifugation and the lipoproteins isolated. The lipophorin samples were further subjected to Sephadex G-75 gel permeation chromatography to separate lipophorin from unbound apoLp-III. The lipoprotein fractions obtained were then electrophoresed on a 4-15 % acrylamide gradient slab gel. The position and molecular weight of marker proteins is shown at the left. Lane 1) VHDLp fraction isolated following incubation with LTP; 2) HDLp-A from the control incubation; 3) LDL isolated following incubation with HDLp-A and LTP and 4) LDL from the control incubation.

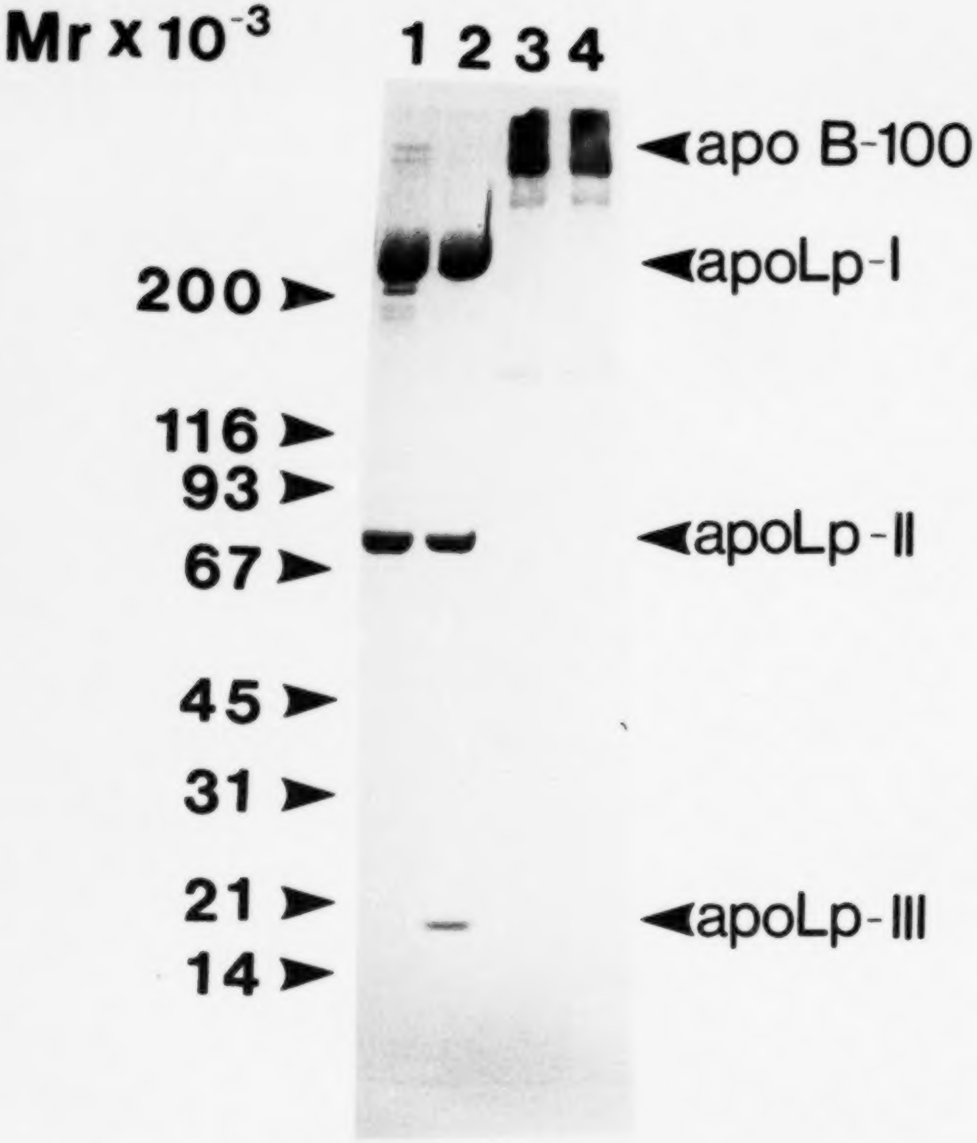

Mr x 10^{-3}
1 2 3 4
200
116
93
67
45
31
21
14
apo B-100
apoLp-I
apoLp-II
apoLp-III

Figure 2.2

Figure 2.3. SDS-PAGE of egg LTP. *M. sexta* eggs were harvested from adult females, homogenized and the buffer soluble protein fraction subjected to a density gradient ultracentrifugation, gel permeation chromatography and ion exchange chromatography as outlined in Materials and Methods to isolate egg LTP. The sample obtained was then electrophoresed on a 4-15 % acrylamide gradient slab gel for 3.5 h and stained with Amido Black 10B. Lane 1) molecular weight markers including myosin, ß-galactosidase, phosphorylase b, bovine serum albumin and ovalbumin. Lane 2) Egg LTP.

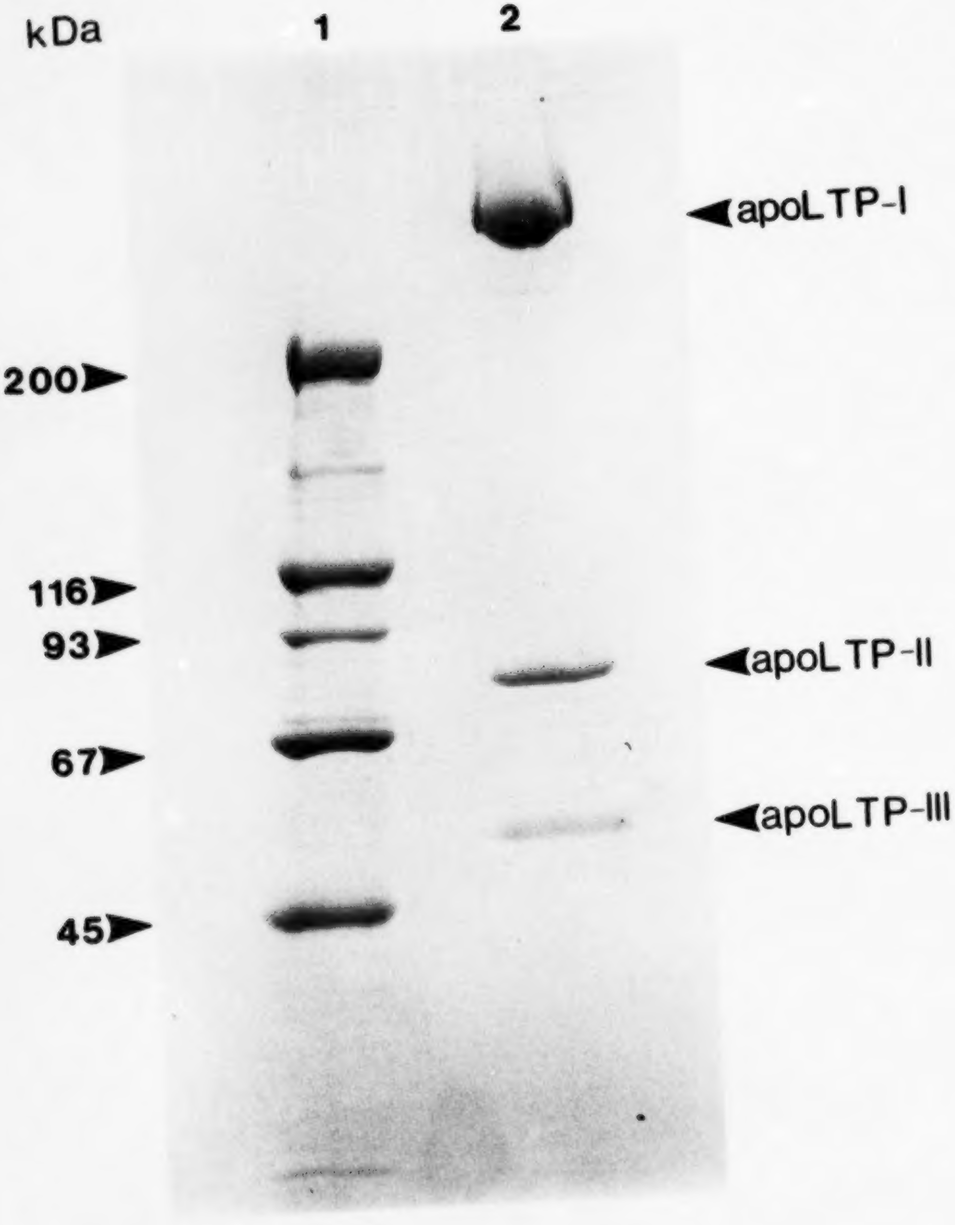


Figure 2.3

Figure 2.4. Double radial immunodiffusion of LTP samples. *M. sexta* hemolymph LTP (1) and egg LTP (2) were diffused against anti hemolymph LTP IgG (3) for 24 h at 25° C in a 0.8 % agarose gel. Following diffusion the gel was washed successively with PBS and H_2O, dried and stained with Coomassie Brilliant Blue.

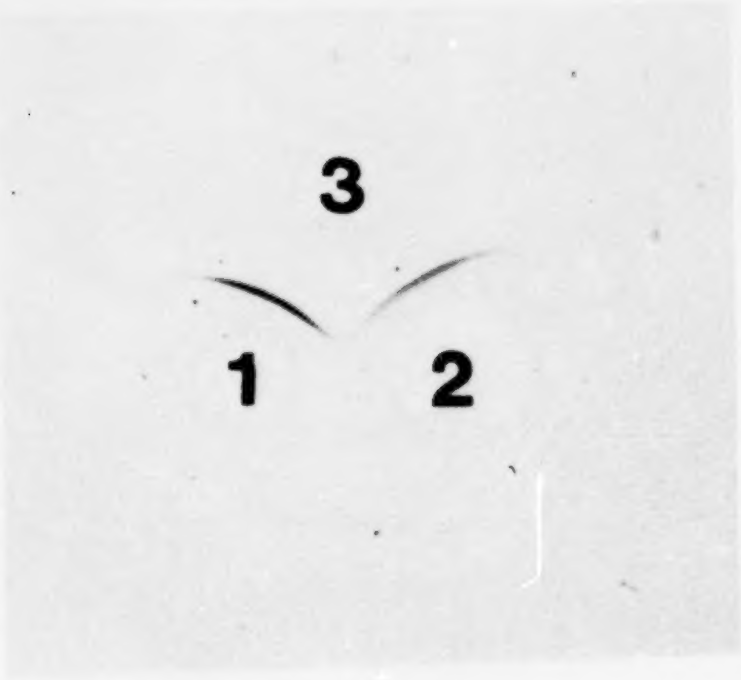
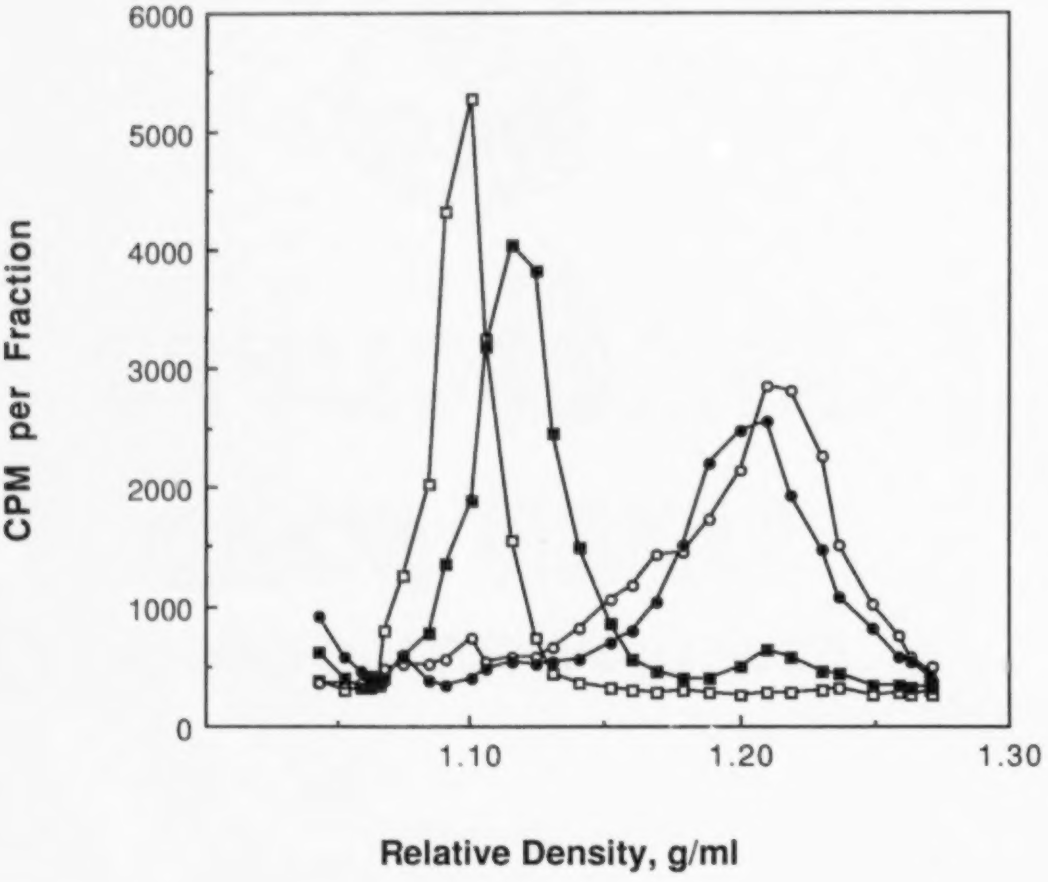


Figure 2.4

Figure 2.5. Effect of anti-hemolymph LTP IgG on transformation of HDLp-A to VHDLp-E by an *M. sexta* oocyte homogenate. About 100 freshly harvested oocytes were homogenized in 1.5 ml of buffer. Tube 1 contained 0.7 ml buffer and tube 2, 3 and 4 contained 32 mg buffer soluble oocyte protein in 0.7 ml buffer. At time zero, 0.5 ml of buffer (tube 1), preimmune serum (tube 2) or anti-LTP Ig-G solution (3 mg, tubes 3 and 4) was added. Incubation was carried out at room temperature for 3.5 hr before adding 7 µg of ^{125}I-HDLp-A (5.3 x 10^3 cpm/µg protein) to each tube. LTP (50 µg) was then added to tube 4 and the tubes were then incubated further for 10 hr. After incubation, the samples were adjusted to a density of 1.31 g/ml by the addition of solid KBr, placed in 39 ml Beckman Quick-Seal tubes, overlayered with 0.9% NaCl and centrifuged (VTi 50 rotor, 4° C for 4 h at 50,000 rpm). After centrifugation the tubes were fractionated from top into 1.3 ml fractions and the density and radioactivity in each fraction determined. Tube 1, *open squares*; Tube 2, *open circles*; Tube 3, *closed squares* and Tube 4, *closed circles*.

Fig. 2.5



6000
5000
4000
3000
2000
1000
0
CPM per Fraction
1.10
1.20
1.30
Relative Density, g/ml

Chapter 3

Human apolipoprotein A-I liberated from high-density lipoprotein without denaturation

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Introduction

As the major protein component of high density lipoprotein (HDL), apolipoprotein A-I (apoA-I) plays an important role in lipoprotein metabolism by stabilizing HDL particle structure and as an activator of lecithin:cholesterol acyltransferase (LCAT) (Eisenberg, 1984). Human apoA-I is a single polypeptide chain composed of 243 amino acids lacking cysteine (Brewer *et al.*, 1978). The lipid binding domain of apoA-I is proposed to consist of multiple segments of amphiphilic α-helix 20-22 amino acids in length (Fitch, 1977, Mclachlan, 1977). In human plasma, although the bulk of apoA-I is found associated with HDL particles, a distinct pool of free apoA-I is thought to exist in equilibrium with lipoprotein-bound apoA-I (Pownall *et al.*, 1978).

ApoA-I is conventionally isolated from HDL by extraction of particle lipids with organic solvents (Scanu & Edelstein, 1971), solubilization of apolipoproteins in urea or guanidine HCl followed by one or more chromatographic steps (Scanu *et al.*, 1969; Shore & Shore, 1969; Reynolds & Simon, 1974). These procedures require that the protein be denatured during isolation. The fact that apoA-I has been characterized after removal of denaturants may cause some skepticism about the physiological relevance of this data. In addition, it is known that prolonged exposure to 6-8 M urea during chromatographic steps can lead to carbamylation of amino groups resulting in artifactual heterogeneity (Gerding *et al.*, 1971; Hagel *et al.*, 1971). Nevertheless it is generally assumed that, upon removal of chaotropic agents by exhaustive dialysis, apoA-I refolds to a conformation similar to that of the pool of free apoA-I in plasma.

We have been studying the properties of a novel lipid transfer particle (LTP) discovered in insect hemolymph (see Ryan, 1990 for review). LTP is a high molecular weight very high density lipoprotein that is proposed to mediate the transfer of lipid between cell membrane and lipoprotein *in vivo* (Van Heusden and Law, 1989). Consistent with this postulated function is the capacity of LTP to catalyze vectorial net transfer of lipid mass among donor/acceptor lipid particles (Ryan *et al.*, 1986; Ando *et al.*, 1990; Ryan *et al.*, 1990a; Liu and Ryan, see previous Chapter) which can result in dramatic alteration of their lipid content and composition. When human HDL is employed as a substrate in the absence of other lipid donor/acceptor particles, LTP induces a dramatic transformation of HDL into larger, less dense lipid particles (Silver *et al.*, 1990). As a result there is a significant reduction in the overall ratio of particle surface area:core volume between substrate HDL and transformation product. Therefore, at equilibrium surface components (phospholipid, unesterified cholesterol, apolipoprotein) in excess of those required to stabilize the product particles are present in the reaction mixture. As a result significant quantities of apolipoprotein dissociate from the surface of the product lipoprotein particles and can be recovered as lipid-free apolipoprotein. Since apoA-I has less affinity for lipid surfaces than apoA-II or the C apolipoproteins (Rosseneu *et al.*, 1976; Lagocki and Scanu, 1980) it preferentially dissociates. Thus apo A-I liberated as a result of this reaction is representative of the pool of free apoA-I proposed to exist *in vivo* and function as a reservoir of apolipoprotein and as a precursor of lipoprotein species that serve as acceptors of cellular unesterified cholesterol in the reverse cholesterol transport pathway (Hara and Yokoyama, 1991).

Materials and Methods

ApoA-I and LCAT purification. HDL was isolated from fresh human plasma between the density limits 1.063 and 1.21 g/ml by sequential flotation ultracentrifugation. LTP was isolated from hemolymph of the tobacco hornworm, *Manduca sexta*, as previously described (Ryan *et al.*, 1990b). To prepare apoA-I HDL was incubated with LTP (20 µg LTP/mg HDL protein) for 120 min in 0.10 M sodium phsophate, pH 7.0, 150 mM NaCl (phosphate buffered saline; PBS) at 37°C. After incubation the sample was adjusted to 1.23 g/ml by the addition of solid KBr and subjected to density gradient ultracentrifugation for 16 h at 40,000 rpm in a Ti 70.1 rotor. The top yellow fraction, containing HDL transformation product and LTP, was removed with a syringe and the infranatant was collected from the bottom. Following dialysis the infranatant was incubated with HDL (1 mg HDL protein per 10 mg bottom fraction protein) for 60 min at 23 °C and then subjected to ultracentrifugation as described above. The bottom fraction was collected, dialyzed against buffer for storage at 4°C or versus deionized $H_2 0$ and lyophilized. For some experiments apoA-I was isolated from human HDL fraction by delipidation and ion-exchange chromatography in 6 M urea as previously described (Scanu *et al.*, 1969; Yokoyama *et al.*, 1982). An aqueous solution of apoA-I isolated by this method was prepared from the lyophilized sample by dissolving in 6 M guanidine HCl and dialysis versus buffer.

LCAT was isolated from human plasma that was subjected to ultracentrifugation at a density of 1.21 g/ml. The clear middle fraction obtained from this centrifugation was dialyzed into 10 mM Tris, pH 7.4, 50 mM NaCl and subjected to Affi-Gel Blue (Bio-Rad) column chromatography where LCAT does not interact with the gel. LCAT containing fractions were pooled and subjected to ion exchange chromatography (DE-52; Whatman) and bound

LCAT was eluted with an NaCl gradient from 75-200 mM. Following hydroxylapitite chromatography in 5 mM sodium phosphate, pH 7.0, where activity is recovered in the flow through fraction, a purified LCAT preparation (2600 times versus plasma) was obtained that was not contaminated by apolipoproteins. The active fractions were pooled and concentrated five fold by ultrafiltration following the addition of bovine serum albumin (0.45 %) and used for experiments.

Analytical ultracentrifugation. ApoA-I samples were dialyzed against 10 mM Tris, pH 7.4, 100 mM KCl for 24 h at 4°C prior to ultracentrifugation. A Beckman model E analytical ultracentrifuge equipped with electronic speed control system, RTIC temperature control system, and titanium rotor was used for all runs. Determination of molecular weights was made using the photoelectric scanner according to the method described by Chervenka (1970). Samples (120 μL) were loaded into a 12 mm double-sector charcoal filled Epon cell equipped with sapphire windows. Runs were performed at 20 °C at speeds ranging from 12,000 to 20,000 rpm for a minimum of 48 h before equilibration photographs were taken. Molecular weight calculations were carried out using a computer program written in APL language. The lnY versus r^2 data were fitted to a 2nd degree polynomial equation using the least squares technique and the point average molecular weights were calculated from the slope of this equation.

Circular Dichroism. Circular dichroism (CD) was performed on a Jasco J-500C spectrapolarimeter with DP500N data processor and thermostatted cell holder with a water bath at 25°C. The cells were 0.05 cm. The instrument was calibrated with d(+)10 camphor sulphonic acid at 290 nm and with pantyllactone dissolved in water at 219 nm. Ten scans were performed on each sample in addition to the appropriate blanks. The data were plotted as mean residue weight ellipticity expressed in degrees cm^2 per decimole versus wavelength in nanometers. The mean residue weight was taken as 117.0. The ellipticity versus wavelength data were analyzed by a computer program developed by Provencher & Glöckner (1981) which analyzes CD spectra as a sum of 16 proteins, the structures of which are known from X-ray crystallography. The input to the program was the mean residue ellipticities in 1 nm intervals from 190 nm to 240 nm.

LCAT assay. Small unilamellar vesicles of egg phopshatidylcholine (PC)/cholesterol (4/1, mol/mol) (obtained from Avanti and Sigma, respectively) were prepared by sonication and used as a substrate (Yokoyama *et al.*, 1980). Assays mixtures (230 µl) contained 40 µl of the LCAT preparation, 100 nmol PC, 25 nmol unesterified cholesterol, 0.75 µCi [7(n)-^{3}H]cholesterol (Amersham), 1.8 mg bovine serum albumin, 40 nmol ß-mercaptoethanol, 7 nmol EDTA and a given concentration of human apoA-I. All solutions were prepared in 10 mM sodium phosphate pH 7.4, 150 mM NaCl. Incubations, conducted for 2.5 h at 37°C, were terminated by adding 2.75 ml chloroform:methanol (2:1) and extraction of the lipids. Lipid extracts were subjected to thin layer chromatography on glass plates precoated with silica gel 60 and developed in hexane:diethylether: acetic acid (80:20:1). The cholesterol and cholesterol ester bands were scraped and the radioactivity counted in a Beckman LS 6000 TA liquid scintillation spectrometer.

Analytical methods. SDS-PAGE was performed on 8-18 % acrylamide gradient slabs electrophoresed at 30 mA for 3.5 h and stained with Coomassie

Brilliant Blue. The content of choline-containing phospholipids and unesterified cholesterol in affinity-isolated apoA-I was determined by commercial enzyme-based colorimetric assays (Boehringer).

Results

Affinity isolation of apoA-I. When incubated with catalytic amounts of insect LTP, human HDL undergoes a dramatic transformation reaction which results in the formation of larger, less dense product lipoproteins that shed apolipoproteins (Silver *et al.*, 1990). In the case of HDL_3 up to 70 % of the apolipoprotein dissociates from the particle surface. As such this reaction is clearly distinct from ultracentrifugation-induced stripping of apoA-I from HDL in the presence of salt. Lipid poor apoproteins obtained by LTP-mediated lipid transfer are comprised predominantly of apoA-I with lesser amounts of apoA-II and C apolipoproteins. Based on the differential affinity of these apolipoprotein components for lipid surfaces it was possible to remove apoA-II as well as the C apolipoproteins by incubation with isolated HDL resulting in a competition for the limiting available lipid surface. Under these conditions ApoA-II and C apolipoproteins present as minor contaminants displace apoA-I from the surface of HDL (Rosseneu *et al.*, 1976; Lagocki and Scanu, 1980) and subsequently float with HDL when subjected to ultracentrifugation at a density = 1.23 g/ml. The resulting bottom fraction contains apoA-I as the sole protein component (Figure 1). Lipid analysis of isolated apoA-I revealed < 1 % by weight phospholipid and cholesterol as contaminants in our preparation indicating that under these conditions, phospholipid and cholesterol moieties do not dissociate from the HDL particle surface and that among the apolipoproteins, apoA-I does not compete effectively with apoA-II or C apolipoproteins for available surface. In contrast to all previous methods reported, isolation of apoA-I by this affinity technique does not employ denaturants or solvent extraction to remove lipid and denaturation/renaturation of the apolipoprotein is not required. Since it is not known what effect denaturation has on apoA-I we characterized the apolipoprotein and compared its properties with apoA-I isolated by conventional techniques involving organic solvent extraction of HDL lipids and chromatography of resolubilized apolipoproteins in 6 M urea.

Hydrodynamic properties of affinity purified apoA-I. Sedimentation equilibrium experiments were conducted to assess the self association properties of affinity isolated apoA-I. A plot of apparent molecular weight versus concentration (Figure 2) showed a range of molecular weights from 30,000 to 100,000 consistent with the presence of monomeric species as well as dimers and tetramers. These results are in agreement with the behavior of apoA-I isolated by conventional delipidation/denaturation methods (Vitello and Scanu, 1976, Formisano *et al.*, 1978; Yokoyama *et al.*, 1982) and indicates self association of lipid-free apoA-I is an intrinsic property of the apolipoprotein rather than an artifact induced by denaturation/renaturation. The tendency to self associate when not bound to lipid surfaces may provide a means whereby hydrophobic residues involved in interacting with lipid surfaces are shielded from the aqueous environment. From X-ray crystallography, it has been shown that helical segments in other water soluble apolipoproteins form intramolecular bundles which shield hydrophobic residues from the aqueous environment when not bound to a lipid surface (Breiter *et al.*, 1991; Wilson *et al.*, 1991). Results with apoA-I, on the other hand, may suggest a similar intramolecular arrangement of helical segments does not form, thereby inducing dimerization/oligomerization.

Circular Dichroism Spectroscopy. It is known that urea or guanidine HCl treatment employed in conventional methods of apoA-I isolation results in loss of secondary structure and in this solvent apoA-I has little if any organized structure (Lux *et al.*, 1972). Upon removal of urea or guanidine apoA-I refolds with the return of secondary structure with a helical content 20-30 % lower than apoA-I bound to HDL. It has been postulated that these spectral differences may reflect changes in length, tightness or orientation of the different helical segments of the polypeptide. On the other hand, these alterations could be due to incomplete or improper refolding of apoA-I upon removal of chaotropic agents. Thus we compared the spectral properties of apoA-I prepared without denaturation with those reported earlier for apoA-I isolated by conventional methods. ApoA-I contains 4 tryptophans, 7 tyrosines and 6 phenylalanines. The CD aromatic region, as shown in the inset to Figure 3, is very similar to an earlier study by Lux *et al.* (1972) on apoA-I prepared by a denaturation step. There are minima at 293, 268 and 262.8 nm, maxima at 281, 277.1, 268 and 259 nm, with a shoulder at 302 and 288.3 nm. The tryptophans will be responsible for all the peaks and troughs above 280 nm, while the 277.1 nm band is due to the tyrosine residues and the other bands in the lower wavelength region, to the phenylalanine residues.

The far UV spectrum for apo A-1 prepared by the affinity method has minima at 221 and 208 nm with a peak at 190 nm, all indicative of a helix containing protein, in agreement with earlier CD studies carried out on material prepared by denaturation/renaturation procedures (Scanu, 1965; Lux *et al.*, 1972; Jackson *et al.*, 1973; Yokoyama *et al.*, 1982). The negative ellipticity at 221 nm in the present study and the earlier investigations is ~-15000°, which results in comparable α-helical content estimates (50-55%). Provencher-Glöckner analysis of the spectrum in Fig. 3 reveals 51% α-helix, 26% ß-sheet, 19% ß-turn and 4% random coil. Trifluoroethanol, known to have a ß-sheet and α-helix stabilizing effect by inducing formation of intramolecular hydrogen bonds and/or non-polar interactions (Greff *et al.*, 1976), induced substantial increases in the far UV ellipticities: the $[\theta]_{190\ nm}$ value increases by 22%, the $[\theta]_{208\ nm}$ value by 28% and $[\theta]_{220\ nm}$ value by 24%. These changes, when analyzed by the Provencher-Glöckner program, reveal 69% α-helix, 18% ß-sheet, 9% ß-turn and 4% remainder, resulting in the induction of some 20% additional helix in apo A-1 upon the addition of trifluoroethanol.

Activation of LCAT. The ability of affinity isolated apoA-I to activate LCAT was determined by incubation of apoA-I with PC/cholesterol unilamellar vesicles and partially purified LCAT. The results showed (Figure 4) a concentration dependent activation of LCAT that was similar to that of apoA-I isolated by conventional methods. As indicated in the inset, the rate of activation was proportional to the bound apoA-I, calculated using K_d (3.0×10^{-7} M) and maximum binding (3.7×10^{-3} mol apoA-I/mol PC) levels obtained from equilibrium binding experiments of the protein previously measured with this type of vesicle (Yokoyama *et al.*, 1980). The data are consistent with previous studies of LCAT activation by apoA-I as well as synthetic model peptides (Yokoyama *et al.*, 1980, Fukushima *et al.*, 1980). These results suggest that apoA-I which dissociates from lipoprotein surfaces as a function of lipoprotein metabolism likely resides in plasma as a reserve capable of reassociation with lipid surfaces and LCAT activation. Furthermore the fact that apoA-I isolated by denaturation/renaturation was similar to affinity isolated apoA-I in these LCAT activation studies provides support for the concept that, following denaturation, apoA-I refolds to a conformation very similar to that of native lipid-free apoA-I.

Discussion:

Insect hemolymph LTP is a remarkable catalyst that can induce redistribution of lipoprotein-associated lipid via facilitated vectorial net lipid transfer. It is thought that LTP functions to facilitate establishment of an equilibrium distribution of the lipid components of substrate lipid particles. In addition, when present, exchange or transfer of low molecular weight, water soluble apolipoproteins may occur (Ryan *et al.*, 1990c). A consequence of this redistribution, however, is potential alteration of the surface area:core volume ratio in the substrate particles versus the products. When this ratio decreases excess surface components are free to dissociate from the lipoprotein surface. Such a reaction has been observed to occur when human HDL is incubated with catalytic amounts of LTP. We have used this LTP-induced diminution of available surface to isolate water soluble, lipid-free HDL apolipoproteins. We further suggest that this reaction represents an *in vitro* model of a general phenomenon in lipoprotein metabolism whereby alterations in lipoprotein composition induced by enzymes such as hepatic lipase, lipoprotein lipase or LCAT result in apolipoprotein dissociation/association to maintain a stable particle structure. To our knowledge this is the first confirmed report of adaptation of this phenomenon to liberation of lipid-free apolipoproteins. We have found that apoA-I can be selectively isolated in a lipid-free, water soluble form by incubation of dissociated apoproteins with a limiting quantity of HDL. Since apoA-I has a lower affinity for lipid surfaces than either apoA-II or the C apolipoproteins (Rosseneu *et al.*, 1976; Lagocki and Scanu, 1980), these latter apoproteins effectively compete for available lipid surface on the added HDL and displace apoA-I. Subsequent flotation of the HDL results in recovery of apoA-I in the bottom fraction. While at the present time broad application of this procedure is limited by the availability of large amounts of LTP, an attractive feature of this reaction is that, unlike all previous isolation procedures, apoA-I denaturation is not required in this isolation scheme. Thus we are confident that its conformation in solution accurately reflects that of the pool of lipid-free apoA-I postulated to exist *in vivo* (Eisenberg, 1984, Pownall *et al.*, 1978).

An important property of apoA-I that could result from denaturation/renaturation is a tendency to self associate into multimeric structures. When we examined the behavior of affinity purified apoA-I in sedimentation equilibrium experiments evidence of self association was obtained suggesting this property is not an artifact of denaturation/renaturation but rather is an intrinsic characteristic of lipid-free apoA-I. It is likely that this behavior may reflect a tendency of the hydrophobic regions of a given apoA-I to interact with the hydrophobic region of a second apoA-I, thereby shielding these residues from the aqueous environment. In contrast to this scenario is the proposed folding of water soluble apolipoproteins which are not prone to self association, such as insect apolipophorin III and the N-terminal region of human apoE (Kawooya *et al.*, 1986, Aggerbeck *et al.*, 1988), into helical bundles whereby hydrophobic residues face inward and hydrophilic residues face outward (Breiter *et al.*, 1991, Wilson *et al.*, 1991). This structural motif implies that the apolipoprotein unfolds to expose its hydrophobic residues upon binding to a lipid surface. Whether apoA-I may possess a similar structural motif will require additional structural information.

The near and far UV CD spectra for apoA-I prepared by the affinity method is qualitatively similar to that of apoA-I prepared by the denaturation/renaturation procedure reported in earlier studies cited above. Provencher-Glöckner analysis reveals some 50% α-helix in the affinity purified material, comparable to the value observed for denatured-renatured preparations, as well as the presence of some 26% ß-structure and 19% ß-turn. Trifluoroethanol induces some 20% additional α-helix at the expense of ß-structure in affinity prepared apoA-I, an effect parallelling the observed induction of 20-30% α-helix in the denatured-renatured material by the addition of phospholipid and CE (Lux *et al.*, 1972). However, it is to be recognized that these figures may be misleading in light of recent X-ray crystallographic studies on functionally related apolipoproteins, locust apolipophorin III and a 22 kDa fragment of apoE. Apolipophorin III has an overall molecular architecture of five long α-helics connected by short loops with some 84% of the residues in the α-helical conformation (Breiter *et al.*, 1991), and the receptor binding domain of human apo-E is a four helix bundle, with 62% of its residues in the α-helical conformation (Wilson *et al.*, 1991). Of relevance to the CD analysis is the fact that a major limitation of the Provencher-Glöckner program, or any other current method used in the analysis of the various conformations of a protein from its CD data, is the choice of the reference proteins in the data base that will represent adequately the protein to be analyzed. Of the 16 reference proteins used in the Provencher-Glöckner analysis only 2 (myoglobin & lactate dehydrogenase) have substantial amounts of helix. Most certainly with apolipoproteins, the data base should be expanded to include those whose crystal structures have been determined, particularly in view of their high helical content.

The ability of affinity purified apoA-I to activate LCAT was very similar to that of apoA-I that had been previously denatured-renatured and the levels of activation for both preparations was proportional to the apoA-I bound to the substrate vesicles (Yokoyama *et al.*, 1980, Fukushima *et al.*, 1980). This result indicates that denaturation-renaturation of apoA-I does not compromise its ability to activate this enzyme and indicates that its interaction with the substrate lipids is not significantly different from that of the affinity isolated apoA-I.

Lipid transfer protein in mammalian blood plasma catalyzes random and non-directional exchange reaction of the lipoprotein core lipids, mainly CE and TG, and does not seem to catalyze net lipid transfer between lipoprotein subfractions (Morton & Zilversmit, 1983, Nishikawa *et al.*, 1988). However, Barter and colleagues recently reported that, in the presence of free fatty acid, human plasma lipid transfer protein catalyzes net lipid movement from HDL to low density lipoprotein (Newnham and Barter, 1990; Barter *et al.*, 1990a, b) or conversion of HDL to large particles leaving a very small lipoprotein complex (Barter *et al.*, 1990b, Lagrost & Barter, 1991). Furthermore, these authors have suggested the possible generation of lipid-free apolipoproteins from HDL by this reaction (Barter, 1991). Thus, this reaction may be analogous to that catalyzed by insect LTP which results in apoA-I liberation from the surface of HDL. Such a pool of free apolipoprotein may also function physiologically by interacting with, and accepting lipid from, peripheral cells to generate HDL like particles with concomitant reduction of intracellularly accumulated CE (Hara & Yokoyama, 1991). Since the K_m value of this reaction is very low (ie. 1/400 of plasma apo A-1), it is entirely possible that lipid free apolipoprotein can be generated in processes similar to those described in this paper or by Barter and colleagues, and thereby play a key role in the first step of the reverse cholesterol transport pathway.

In sum we have shown that alteration of the surface area: core volume ratio of lipoproteins can be used as an effective method to prepare lipid free apolipoproteins that may be further purified on the basis of their relative affinities for lipid surfaces. Whereas the affinity of certain apolipoproteins for lipid surfaces has been used in isolation procedures for many years (apoA-IV binding to intralipid; Weinberg & Scanu, 1983) the present method offers the first example of affinity based dissociation of apolipoprotein from lipoprotein surfaces due to a decrease in available lipid surface. Thus apoA-I prepared by this method is suitable for use in lipoprotein reconstitution, LCAT assays and physicial characterization. Furthermore it is possible that apoA-I isolated by this method may be more amenable to crystallization, thereby permitting further structural characterization. The results also provide validation of the assumption that denaturation steps commonly employed in apolipoprotein isolation are completely reversible.

This Chapter we purified apo A-I based on its relative lipid binding affinity compared with other human apolipoproteins. Next Chapter we will examine the relative lipid binding affinity of apo A-I obtained by this method with that of insect apoLp-III in LDLp particle.

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Figure 3.1. SDS-PAGE of affinity isolated apoA-I. A-8-18% acrylamide gradient slab loaded with 25 g apo A-I was electrophoresed at 30 mA constant current for 3.5 h. The position of molecular weight standards including phosphorylase b, albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme are shown.

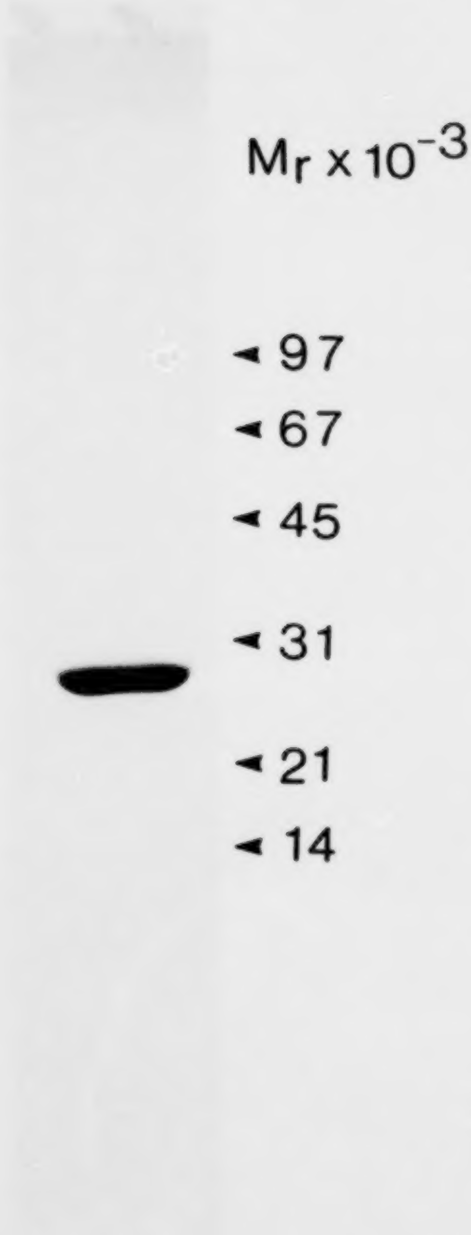


Figure 3.1

Figure 3.2. A plot of the apparent molecular weight (M_{app}) versus concentration for apoA-I prepared by the affinity method. The solvent system was 10 mM Tris and 100 mM KCl at pH 7.4. The centrifuge speed was 15,000 rpm at 20 C.

Fig. 3.2

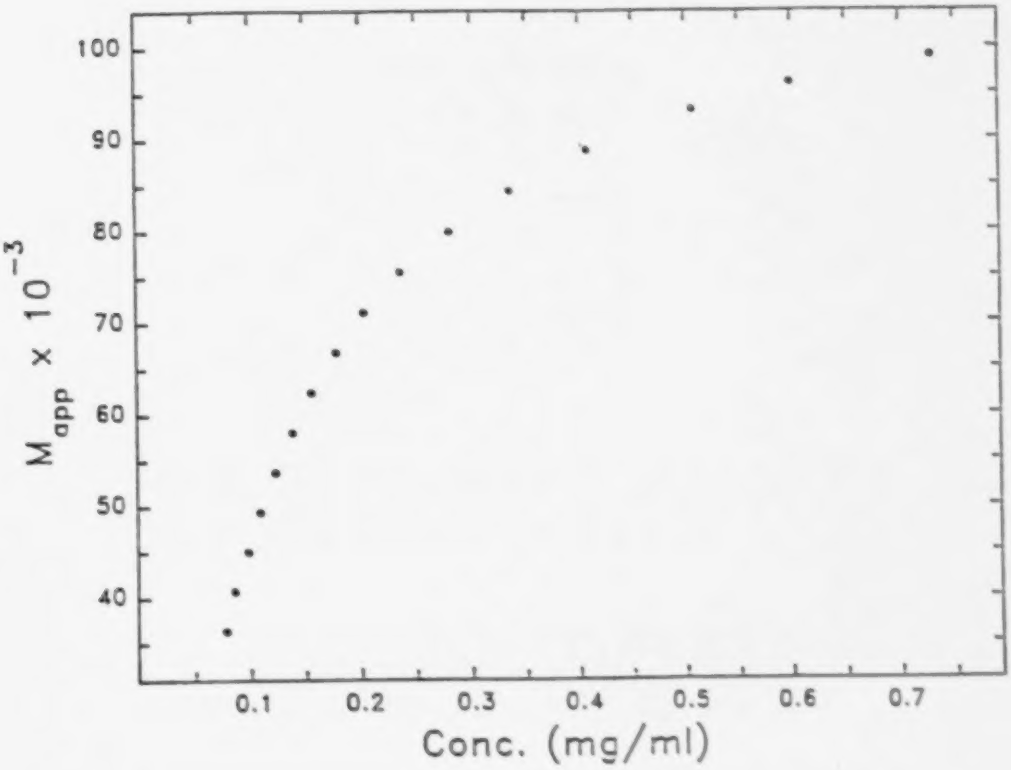


Figure 3.3. Circular dichroism spectra of apoA-I. Far U.V. spectrum for apoA-I prepared by the affinity method, in 0.1 M KCl, 0>1 M Tris HCl, pH 7.4 (———) and in 50 % TFE, 0.05M KCl, 0.05 M Tris HCl, pH 7.4 (-------). The inset represents the near U.V. spectrum in 0.1 M KCl, 0.1 M Tris HCl, pH 7.4.

Fig. 3.3

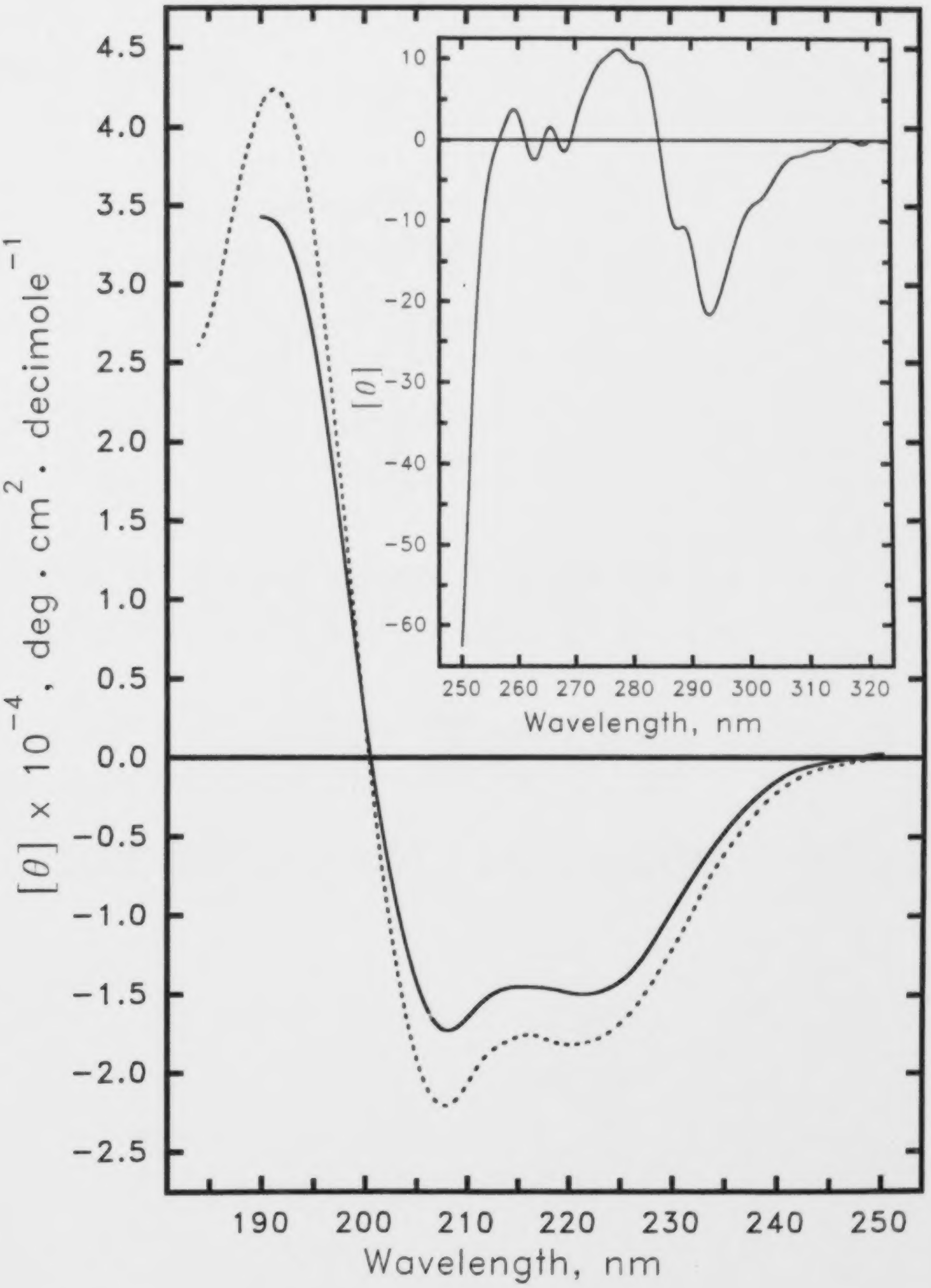
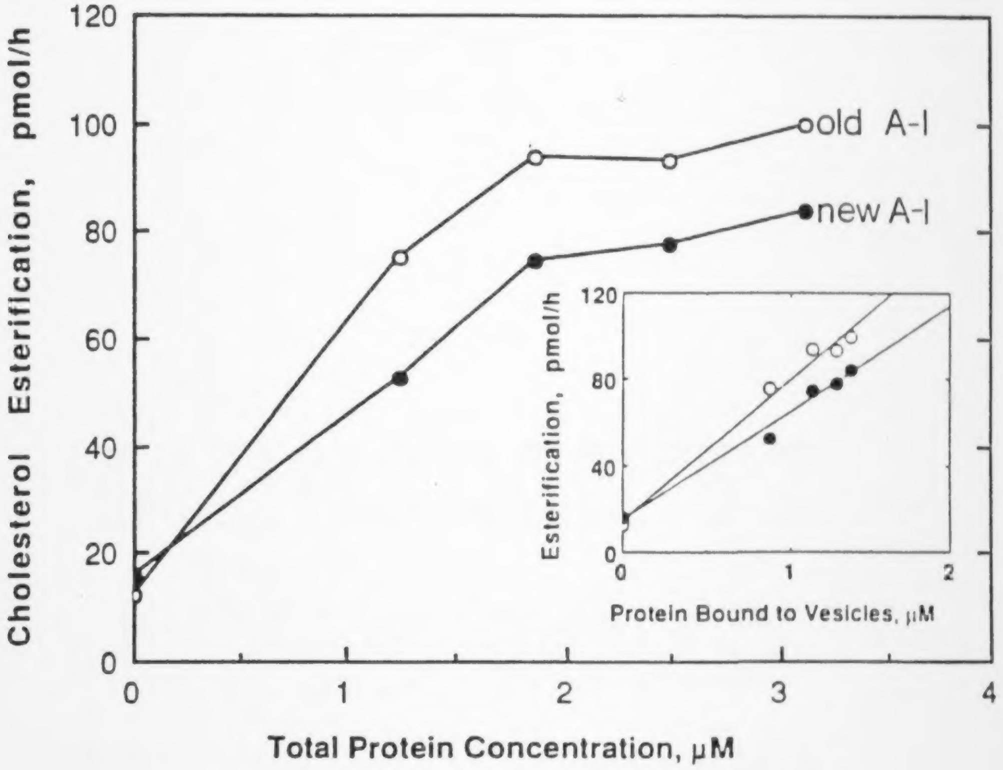

4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
−0.5
−1.0
−1.5
−2.0
−2.5
$[\theta] \times 10^{-4}$, deg · cm^2 · decimole^{-1}
190
200
210
220
230
240
250
Wavelength, nm
10
0
−10
−20
−30
−40
−50
−60
$[\theta]$
250
260
270
280
290
300
310
320
Wavelength, nm

Figure 3.4. Activation of lecithin:cholesterol acyltransferase by human apoA-I on small unilamellar vesicles of PC:cholesterol (4:1, mol/mol) as a functiuon of apoA-I concentration in the reaction mixture. Open symbols are the activation by apoA-I isolated by methods employing denaturation steps and closed symbols represent activation by apoA-I isolated by the affinity method described in the present work. Details of the experimental conditions are described in Materials and Methods. The inset illustrates the same data plotted against the apoA-I bound to the substrate vesicles (calculated according to the equation of equilibrium binding using parameters measured in a previous study; Yokoyama *et al.*, 1980). The straight lines represent least square linear regression of the data.

Fig. 3.4


Cholesterol Esterification, pmol/h
120
100
80
60
40
20
0
0
1
2
3
4
Total Protein Concentration, μM
old A-I
new A-I
Esterification, pmol/h
120
80
40
0
0
1
2
Protein Bound to Vesicles, μM

Chapter 4

DISPLACEMENT OF APOLIPOPHORIN III FROM THE SURFACE OF LOW DENSITY LIPOPHORIN BY HUMAN APOLIPOPROTEIN A-I

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Introduction

Apolipoproteins from mammalian and invertebrate sources can be classified into two categories:1) integral apoprotein, such as apo B-48 and apo B-100 in mammalian systems, and apoLp-I and apoLp-II in insects; 2) surface amphipathic apolipoproteins. Insects that utilize lipid as a fuel for sustained flight possess a novel mechanism for increasing transport of lipid from fat body storage depot to flight muscle. The hemolymph lipoprotein, adult high density lipophorin (HDLp-A), serves as acceptor of fat body lipid and, under the influence of adipokinetic hormone, is transformed into low density lipophorin (LDLp; 1,2). Concomitant with lipid uptake by HDLp-A up to 16 molecules of an abundant, low molecular weight, water soluble protein, apolipophorin III (apoLp-III), associate with LDLp (3-6). Isolated apoLp-III binds phospholipid or DG surfaces with high affinity (8) and it has been postulated that it serves to stabilize the increment of lipid/water interface created by lipid uptake (7). Although the amino acid sequences of *Manduca sexta* (9) or *Locusta migratoria* (10) apoLp-III do not share significant identity with their apparent mammalian counterparts, apolipoproteins A-I, A-II, A-IV, E, D or C, there is evidence of functional homology (9,10). X-ray crystallography of *L. migratoria* apoLp-III revealed a structure rich in α-helix containing five long helices connected by short loops (11). The loops form a bundle in which the hydrophilic residues face outward and the hydrophobic residues face inward. The three dimensional structure of a 22 kDa fragment of human apolipoprotein E has features that are very similar to that of apoLp-III (12) suggesting the elongated helical bundle may be a common structural motif for apolipoproteins.

Based on the apparent structural relationship between insect apoLp-III and human apoE it is of interest to define the functional relationship between apoLp-III and members of the mammalian apolipoprotein family. A variety of systems have been used to study the lipid binding properties of water soluble apolipoproteins including lipid monolayers (13), synthetic phospholipid vesicles (14) and lipoproteins (15,16). We have used LDLp as a model lipid surface to examine the lipid binding affinity of apoLp-III and human apoA-I. Our findings indicate that apoA-I has a higher affinity for the surface of LDLp and displaces apoLp-III to form a stable hybrid LDLp particle.

Material and Methods

Lipoprotein and apolipoprotein isolation. LDLp was isolated from adult *Manduca sexta* hemolymph by density gradient ultracentrifugation as described elsewhere (17). ApoLp-III was isolated according to Wells *et al.* (18). Human high density lipoprotein (HDL) was isolated by sequential KBr density gradient ultracentrifugation between the density limits 1.063 g/ml and 1.21 g/ml and apoA-I was prepared by reacting HDL with *M. sexta* LTP as described by Silver *et al.* (19). ApoA-I was labeled with succinimidyl [2,3-^{3}H]propionate (94.3 Ci/mmol) according to the supplier's (Amersham) instructions.

Displacement studies. Lipoprotein samples were incubated for 90 min at 33 C in the presence or absence of specified apolipoproteins. Following incubation the samples were centrifuged at a density of 1.12 g/ml for LDLp or 1.21 g/ml for HDL in a Ti70.1 rotor for 16 h at 60,000 rpm and the lipoproteins collected from the top of the tubes. Alternatively samples were centrifuged at 50,000 rpm for 4 h at 4 C in a VTi50 rotor as described (4). After centrifugation the tube contents were fractionated and the density, protein and/or radioactivity determined.

Analytical methods. SDS-PAGE was performed on 4-15 % acrylamide gradient slab gels according to Laemmli (20) and stained with Coomassie Brilliant Blue R-250. Protein was determined gravimetrically for isolated, lyophilized apolipoproteins or by the Bicinchoninic assay (Pierce Chemical Co.). Densitometric scanning of stained gels was performed on a Camag TLC scanner II. Electron microscopy of negatively stained lipoprotein samples was performed on a Philips EM420 microscope operated at 100 KV as described (21). The lipid content of native and hybrid LDLps was analyzed by thin layer chromatography and densitometric scanning. Lipids were extracted with 2:1 chloroform: methanol, dried under a stream of N_2 and separated on glass plates precoated with silica gel 60 developed in hexane:diethyl ether:acetic acid 70:30:1 (v/v/v). After chromatography lipids were charred by immersion in 3 % cupric sulfate (w/v)-8% phosphoric acid (v/v) solution followed by heating at 180 C for 15 min.

***In vivo* experiments**. LDLp was iodinated as described by Markwell (22) using $Na^{125}I$ (100 Ci/ml; Amersham) and subsequently 1.5 mg of the labeled lipoprotein was incubated for 60 min with 3 mg of unlabeled apoLp-III or apoA-I to exchange or displace labeled apoLp-III. The samples were then subjected to density gradient ultracentrifugation at 1.10 g/ml KBr in a Ti70.1 rotor at 50,000 rpm for 16 h. The LDLp fractions were recovered and analyzed by SDS-PAGE and autoradiography which indicated that > 95 % of the radiolabel was present in apoLp-I and apoLp-II. Fifty microgram of the ^{125}I-LDLps (specific activity = 6400 cpm/ g protein) were injected into each of two adult

M. sexta and after 16 h the animals were bled. The hemolymph was adjusted to 1.21 g/ml with solid KBr in a volume of 20 ml, overlayered with 0.9 % saline and subjected to vertical rotor density gradient ultracentrifugation (4). The tube contents were then fractionated and the density and radioactivity in each fraction determined.

Results and Discussion

When subjected to density gradient ultracentrifugation, purified, lipid-free, ^{3}H-apoA-I was recovered in the bottom fraction (Figure 1). Incubation with LDLp, however, resulted in recovery of ^{3}H-apoA-I at a relative density of 1.05 g/ml, suggesting apoA-I associated with LDLp. When excess unlabeled apoA-I was added to incubations of ^{3}H-apoA-I and LDLp the bulk of the labeled apoA-I was again recovered in the bottom fraction suggesting its interaction with LDLp is specific. Since the size of apoA-I (M_r =28,000) is distinct from that of the three apolipoprotein components of LDLp (M_r = 240,000, 80,000 and 18,000, respectively) we used SDS-PAGE to characterize the apolipoprotein composition of LDLp as a function of apoA-I concentration (Figure 2). The appearance of apoA-I in fractions corresponding to the density of LDLp was accompanied by a concomitant loss of apoLp-III from the LDLp density fractions in a concentation dependent manner. Increasing the amount of apoA-I in incubations resulted in a corresponding depletion of apoLp-III from the LDLp density range. ApoLp-III lost from the LDLp density range was recovered in the bottom fraction. When an excess amount of apoA-I was incubated with LDLp both apoLp-III and apoA-I were recovered in the bottom fraction indicating that the apparent association of apoA-I with LDLp is saturable. The apoprotein stoichiometry of native and hybrid LDLp particles was quantitated by scanning densitometry of SDS-PAGE gels and comparison with standard curves constructed from known amounts of lipophorin or purified apolipoprotein. In agreement with previous work (7) control LDLp particles had an apoLp-I:apoLp-II:apoLp-III molar ratio of 1:1:16. The sample in lane 2 of Fig. 2 had an apoLp-I:apoLp-II:apoLp-III ratio of 1:1:10 but also contained 3 molecules of apoA-I. In lane 5 where the maximum amount of apoA-I displacement occurred the ratio of apoLp-I:apoLp-II:apoLp-III was 1:1:2 with the lost apoLp-III replaced by 7 molecules of apoA-I. From the apparent stoichiometry of apolipoprotein binding to the surface of LDLp it is evident that one molecule of apoA-I can displace 2 molecules of apoLp-III. This result is consitent with the molecular weights of apoLp-III and apoA-I as well as the known ability of two human apoA-II molecules (M_r = 17,000) to displace one apoA-I from the surface of canine HDL (15). Based on previous results we predict that apoA-II, apoC-I and apoC-III also have a higher affinity for the surface of LDLp than apoLp-III (23).

Interestingly, under conditions where excess apoA-I is present, a discernable amount of apoLp-III continues to float in the LDLp density range. This result is consistent with previous findings (7) that two molecules of apoLp-III present as apoprotein components of the precursor of LDLp, HDLp-A, are nonexchangeable. Furthermore the data provide support for the concept that, although they are indistinguishable on the basis of their physical or immunological properties (24), all of the apoLp-III of LDLp are not equivalent. Similar conclusions have been made with apoA-I on human HDL (25). When hybrid particles were incubated with excess isolated apoLp-III, followed by density gradient ultracentrifugation, no evidence of apoA-I displacement was obtained, indicating displacement is not reversible under these conditions. In other experiments isolated apoLp-III was incubated with human HDL (whose primary apolipoprotein component is apoA-I). Characterization of the density distribution of apolipoprotein showed that, as with the hybrid LDLp, apoLp-III was unable to displace apoA-I from the particle surface. Taken together, the results indicate that apoA-I has a higher affinity for lipid surfaces than apoLp-III and is capable of displacing the bulk of apoLp-III from the surface of LDLp, creating an apoA-I hybrid LDLp.

To determine if association of apoA-I with LDLp induced additional alterations in the particle we compared the properties of native LDLp with those of hybrid LDLp which had an apoA-I:apoLp-III molar ratio of 7:2. Electron microscopy of negatively stained samples revealed that both native and hybrid LDLp were spherical with an average diameter of 22 nm, similar to that previously reported for native LDLp (6,26). The relative densities of native and hybrid LDLp (1.05 g/ml) were the same indicating that hybrid particles have a protein:lipid ratio similar to that of native LDLp. Lipid compositions were compared by densitometric scanning of thin layer chromatographs of native and hybrid particle lipid extracts. The results (data not shown) revealed that the types of lipids, as well as their relative amounts, were the same for both particles and reflected the composition of LDLp reported earlier (17). The results indicate that displacement of apoLp-III from the surface of LDLp by isolated human apoA-I does not affect its size, density or lipid content. Thus, we conclude that apoA-I can effectively substitute for apoLp-III in stabilization of the LDLp particle structure.

The substitution of apoA-I for apoLp-III on the surface of LDLp could conceivably influence its metabolism if apoLp-III is required for recognition by docking proteins, membrane receptors or as an activator of lipophorin lipase (2). In order to monitor the fate of native and hybrid LDLp *in vivo*, radioiodinated native and hybrid LDLp particles were prepared in which the radiolabel was incorporated into apoLp-I and apoLp-II. The radioiodinated

LDLp particles were injected into adult *M. sexta* and, after incubation, the hemolymph was collected and the density distribution of radioactivity determined. The results (Figure 3) show that both native and hybrid LDLp were shifted to a density corresponding to that of HDLp-A, the normal metabolic product of LDLp (6). Thus, substitution of apoA-I for the bulk of apoLp-III on the surface of LDLp does not preclude its metabolism to HDLp-A. Insect hybrid lipoproteins generated using an *in vitro* lipid loading system provided evidence that apoLp-III from *M. sexta* can substitute for *L. migratoria* apoLp-III in LDLp formation and hybrid and native particles were comparable in their ability to serve as substrates for flight muscle lipase (27). The ability to form hybrid LDLp with a mammalian apolipoprotein substituting for insectan apoLp-III provides a novel method to investigate the role of apoLp-III in LDLp assembly. Furthermore the results suggest LDLp can serve as a useful model membrane system for apolipoprotein lipid binding studies.

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Figure 4.1. ApoA-I binding to LDLp. Thirty µg ^{3}H-apoA-I (specific activity = 3100 cpm/µg) was incubated for 120 min in the presence and absence of LDLp (700 µg protein). The samples were then subjected to vertical rotor density gradient ultracentrifugation. After centrifugation the tube contents were fractionated (1.1 ml) and the density and radioactivity (per 200 µl) determined. Closed squares, ^{3}H-apoA-I, no LDLp; closed circles, ^{3}H-apoA-I plus LDLp; open circles, ^{3}H-apoA-I, LDLp plus 900 µg unlabeled apoA-I.

Fig. 4.1

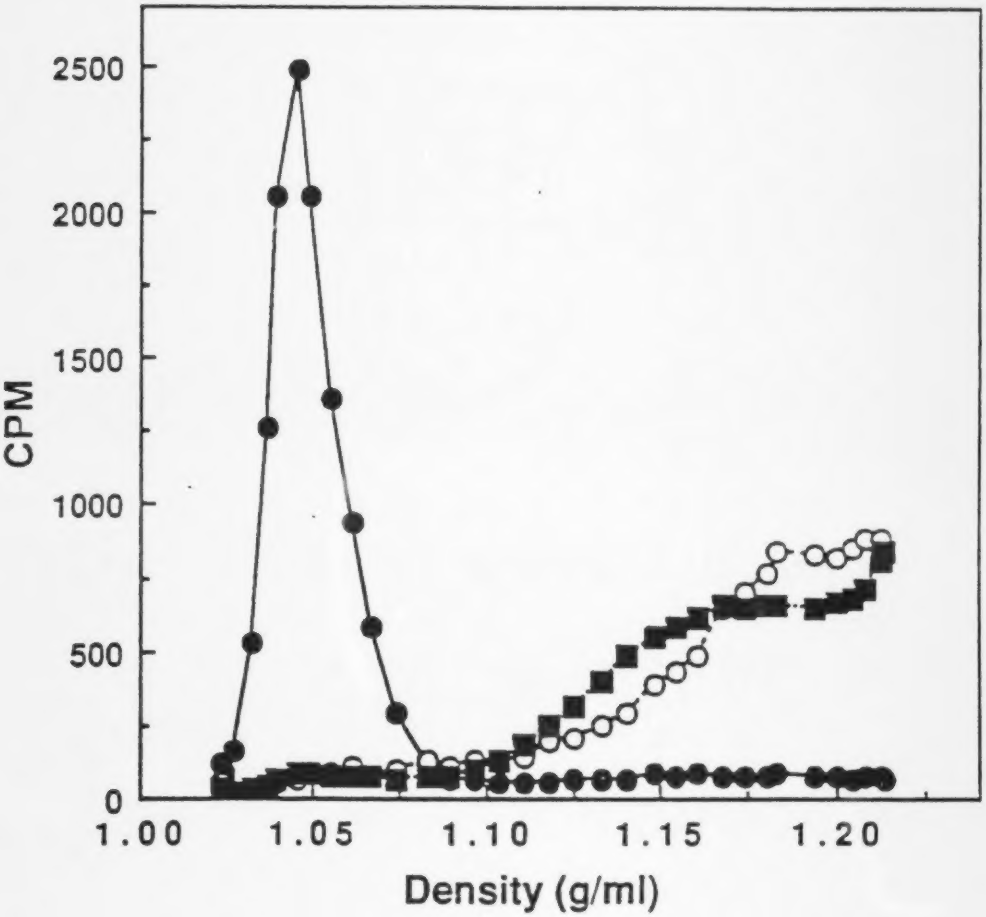


Figure 4.2. SDS-PAGE of native and hybrid LDLp. LDLp (2 mg protein) was incubated with given amounts of apoA-I for 90 min at 33 °C. LDLp was then reisolated by density gradient ultracentrifugation (Ti 70.1 rotor) and its apolipoprotein composition analyzed by electrophoresis under denaturing conditions on 4-15 % acrylamide gradient slab gels. Lane 1) control LDLp (no apoA-I); Lane 2) LDLp plus 0.3 mg apoA-I; Lane 3) LDLp plus 0.75 mg apoA-I; Lane 4) LDLp plus 1.0 mg apoA-I; Lane 5) LDLp plus 2.0 mg apoA-I.

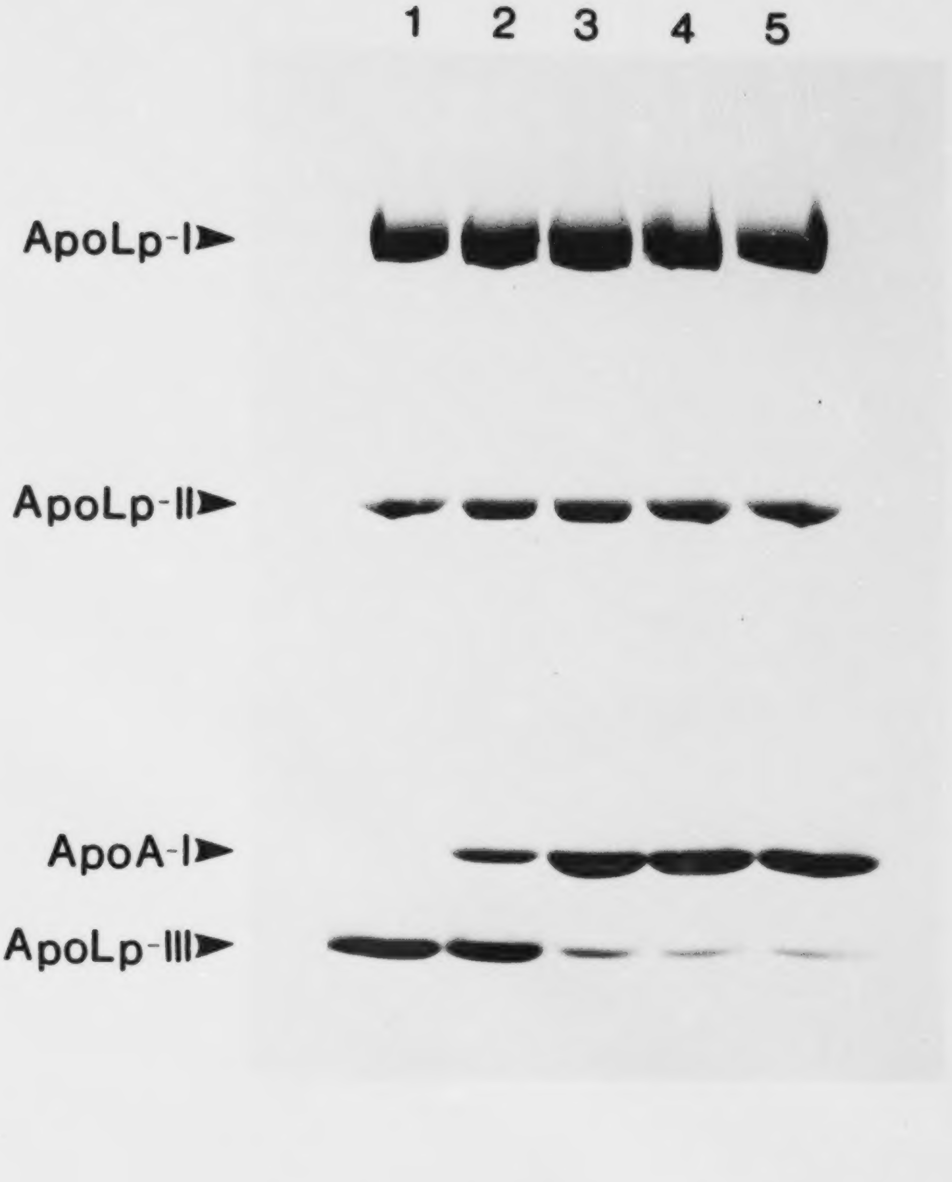
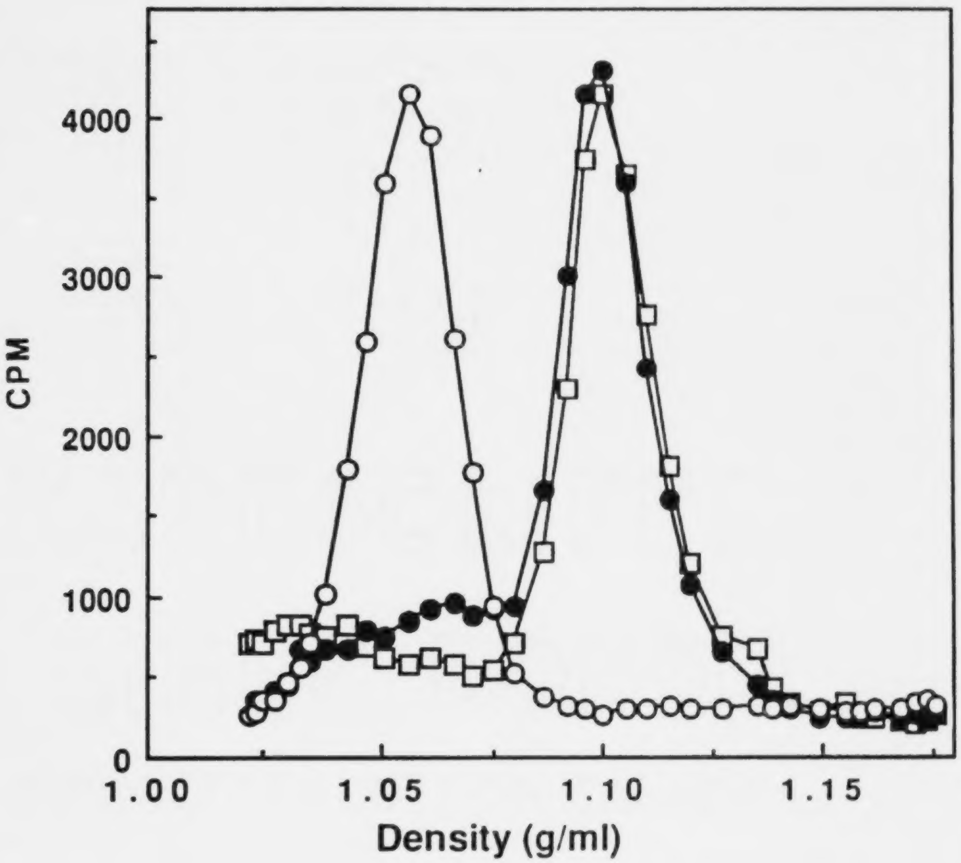


Figure 4.2

Figure 4.3. *In vivo* metabolism of native and hybrid LDLp. Native and hybrid LDLp (labeled in the apoLp-I and apoLp-II moiety with ^{125}I; specific activity = 6400 cpm/µg protein) were injected into each of two adult *M. sexta*. After 16 h the animals were bled and the hemolymph subjected to vertical rotor density gradient ultracentrifugation, fractionated (1 ml) and the radioactivity and density in each fraction determined. Open circles, ^{125}I-LDLp control; Closed circles, density distribution of radioactivity after *in vivo* injection of ^{125}I-native LDLp; open squares, density distribution of radioactivity after *in vivo* injection of ^{125}I-hybrid LDLp.

Fig. 4.3



Chapter 5

Effect of particle lipid content on the structure of insect lipophorins

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Introduction

Hydrophobic lipids are assembled with protein to form a transport carrier within the aqueous medium. In insect hemolymph, high density lipophorin (HDLp) is the major lipid transport vehicle . In the tobacco hornworm, *Manduca sexta* lipophorin can be isolated as distinct subspecies which contain different amounts of lipid while maintaining the same integral apolipoprotein components, apolipophorin I and apolipophorin II (M_r = 240,000 and 80,000, respectively) (1). As discussed earlier, such lipid movement is mediated by LTP *in vivo*. In this chapter we try to study the effect of lipid content, particularly DG, on the structure and morphology of lipophorins.

It has been hypothesized that, like human apo B apolipoprotein, apoLp-I and apoLp-II, together with a small amount of phospholipid, comprise a stable basic matrix structure that is common to all forms of lipophorin (1). The basic matrix particle would therefore have the capacity to accept or donate variable amounts of lipid to produce the different lipophorin subspecies. The isolation and characterization of HDLp subspecies from different developmental stages of *M. sexta* has provided convincing evidence for the versatility of lipophorin with respect to lipid binding capacity (2,3). For all the HDLp subspecies protein constitutes between 48 and 70 % of the particle mass; this being in the range of 500,000 to 800,000 daltons (4). In addition to apoLp-I and apoLp-II, adult high density lipophorin (HDLp-A) possesses two molecules of the low molecular weight apolipophorin III (M_r = 18,000) (5,6). *M. sexta* oocytes (7; Chapter 2) or hemolymph of larvae reared on a fat-free diet (8, Chapter 8) contain a very high density lipophorin (VHDLp) which contains 80 % of its mass as protein in the form of apoLp-I and apoLp-II.

A distinguishing feature of lipophorins is the predominance of DG as their major neutral lipid and a general lack of CE and TG, which are normal core components of mammalian lipoproteins. Although the presence of small amounts of long chain aliphatic hydrocarbon partially compensates for the lack of other nonpolar lipids, difficulties arise when attempts are made to fit the known composition of these particles into structural models developed for mammalian lipoproteins. Compositional analysis of mammalian lipoproteins together with physical measurements and morphological studies fit well with a model in which hydrophobic lipids occupy the particle core and are surrounded by a monolayer of amphiphilic lipid and apolipoprotein(s), generating spherical structures (9). Similar analyses have been performed on lipophorin subspecies (4,10); these are generally consistent with a partitioning of the bulk of the DG as well as some portion of apoLp-II into the interior of the particle. A basic premise of these structural models of lipophorin, however, is that all of the subspecies are spherical. In this communication we present evidence that HDLp subspecies other than HDLp-A are not spherical, and that as the lipid content of the particles decreases the deviation from a sphere-like structure increases. At the same time the sensitivity of apolipophorins to proteolytic digestion and tryptophan fluorescence quenching increases, indicating increasing exposure of apolipoprotein as lipid is removed from the particle.

Materials and Methods

Lipoprotein isolation. HDLp-A, HDLp-W1, HDLp-W2 were isolated from hemolymph and VHDLp-E was isolated from freshly dissected oocytes of the tobacco hornworm, *Manduca sexta*. The insects were obtained from a continuing laboratory colony reared on a wheat germ based diet as described by Prasad *et al.* (2). Hemolymph samples were placed directly into phosphate buffered saline (PBS; 0.1 M sodium phosphate, pH 7.0, 0.15 M NaCl, 1 mM EDTA) containing 5 mM glutathione and 1 mM diisopropylphosphorofluoridate. Hemolymph was centrifuged at 5,000 g for 10 min at 4° C to remove hemocytes, then the density of the solution was adjusted to 1.31 g/ml by the addition of solid KBr. HDLp-A was isolated from 1 or 2 day old adult moths by density gradient ultracentrifugation as described by Ryan *et al.* (3) and HDLp-W1 and HDLp-W2 were isolated from larvae just prior to pupation according to Prasad *et al.* (2) and VHDLp-E was isolated from oocyte homogenates as described by Kawooya, Osir and Law (7).

Electron microscopy. Negatively stained samples (2 % sodium phosphotungstate, pH 7.0) were prepared as previously described (11) and photographed in a Philips EM420 operated at 100 kV.

Circular dichroism. Circular dichroism (CD) measurements were made on a Jasco J-720 spectrapolarimeter (Jasco Inc., Easton MD) interfaced to an Epson 386/25 computer and controlled by software developed by Jasco. The cell was maintained at 25 °C with an RMS circulating water bath (Lauda, Westbury NY). In the wavelength range 255 to 190 nm, cells of path length 0.01 cm were used. The computer-averaged trace of ten scans was used in all calculations and signal due to solvent was subtracted. The instrument was routinely calibrated with d-(+)-10-camphor sulfonic acid at 290 nm, by following procedures outlined by the manufacturer. As a prerequisite to prevent distortion of the CD spectrum, at low wavelengths, the high tension voltage on the photomultiplier was never allowed to exceed 500 V.

The data were normally plotted as mean residue weight ellipticity (expressed in degrees centimeters square per decimole) versus wavelength in nm. The mean residue weight was taken to be 115 for each of the lipoproteins. Lipoprotein concentrations were determined by fringe count in the analytical ultracentrifuge (12) using an average refractive increment of 4.1 fringes per mg protein per ml. A refractive index increment of 0.178 at 546 nm was employed for HDLp-W1 (13), whereas values between 0.178 and 0.185 (100 % protein) were interpolated for HDLp-W2 and VHDLp-E. The concentration of the protein portion of each entity was used in the calculation of molar ellipticities. Since spectra of lipid extracts of HDLp-W1 revealed that the lipid contribution to the CD spectra was negligible, no correction was applied. The ellipticity-versus-wavelength data were analyzed by a computer program (CONTIN) developed by Provencher and Glöckner (14), which analyzes CD spectra as the sum of spectra of 16 proteins, whose structures are known from X-ray crystallography. The input to the program was the mean residue weight ellipiticities, at 1 nm intervals, from 190 nm to 240 nm.

Trypsin digestion. Lipophorin samples were dialyzed against PBS and the protein concentration was adjusted to 1 mg /ml. Fifty microgram of a given lipophorin was then incubated with bovine pancreatic trypsin (Boerhinger) at a trypsin: lipophorin ratio of 1:200 at 25 °C for times ranging from 10 min to 1 h. Control samples were incubated in the absence of trypsin, and the reaction was stopped by placing the samples in SDS sample treatment buffer and immediately boiling for 2 min. For some experiments reactions were stopped by the addition of a two fold molar excess of soybean tryspin inhibitor (Boerhinger), with similar results. The samples were then analyzed by electrophoresis in 4-15 % acrylamide gradient slab gels in the presence of SDS (15).

Fluorescence quenching studies. Flourescence measurements of HDLp-W1, HDLp-W2 and VHDLp-E were performed at 20 °C using a Perkin-Elmer Model 44B spectrofluorometer equipped with a DCSU-2 differential corrected spectra unit and a thermostated cell holder. Measurements were conducted with 1 ml volume Suprasil cells (Hellma) and 5 nm slit widths for both excitation and emission monochromators. Spectra were obtained by using an excitation wavelenth of 295 nm with emission monitored between 325 and 327 nm. Quenching studies were performed by the addition of aliquots of acrylamide in accordance with the methodology described by McCubbin and Kay (16). The fluorescence (F) values were corrected for acrylamide absorption using $F_{corr} = F \cdot 10^{A/2}$,where A is the absorbance in the 1 cm cell at 295 nm.

The fluorescence quenching data were handled via the Stern-Volmer equation: $F_O/F = 1 + K_{SV}[Q]$ where F_O and F represent the fluorescence intensities at the emission maximum in the absence and presence of quencher (Q). The collisional quenching constant, K_{SV} was obtained from the slope of a plot of F_O/F versus [Q].

Results

At least five distinct HDLp subspecies from *M. sexta* hemolymph have been isolated and characterized (4). In addition, hemolymph and oocyte very high density lipophorins have been identified (7,8). All HDLp subspecies, except HDLp-A, contain one molecule each of apoLp-I and apoLp-II as their sole apolipoprotein components; HDLp-A also contains two molecules of apoLp-III. The major difference between the various subspecies is in the amount of DG per particle; this represents from 25 % (HDLp-A) to 20 % (HDLp-W1) to 12.5 % (HDLp-W2) to 4 % (VHDLp-E) of the particle mass. These values correlate with the observed buoyant densities of the individual subspecies, which range from 1.076 g/ml (HDLp-A) to 1.128 g/ml (HDLp-W1) to 1.177 g/ml (HDLp-W2) to 1.238 g/ml (VHDLp-E). The large variations in particle lipid content and density, together with the known interconvertibility of specific subspecies, indicates structural resiliency of the apolipoprotein components. A major unanswered question with respect to lipophorin relates to the effect the above mentioned alterations in particle lipid content have on the structural properties and morphology of lipophorin.

Electron Microscopy. In order to correlate compositional and physical measurements of lipophorins with proposed models of lipophorin structure, the morphologies of isolated particles were examined by electron microscopy. Figure 1 (panel A) reveals that the neutral lipid-deficient VHDLp-E (4 % DG) assumes an asymmetric, crescent-like conformation in which the two ends of the particle are not of uniform size. The lipophorin subspecies, HDLp-W2, which contains three times more DG per particle (12.5 %) than does VHDLp-E but has identical apolipoprotein components, also does not display a spherical morphology. This lipophorin has a U-shaped or cup-like appearance (Figure 1B) and exhibits a more closed conformation than does VHDLp-E. The observed morphologies of these two subspecies demonstrate that the increased neutral lipid content of HDLp-W2 compared to VHDLp-E decreased the overall asymmetry of the particle. This trend is continued with the formation of HDLp-W1 (20 % DG) as shown in Figure 1C. These particles exhibit a roughly rectangular shape with a length of 16 ± 1 nm (n = 100) and a width of 11 ± 1 nm. There is also a cleft which forms a distinct indentation extending from one end of the particle. When the neutral lipid content of particles is increased even further, as in HDLp-A (25 % diacylglcyerol; Figure 1D), a more spherical structure with a diameter of 15 ± 1 nm is formed. The particle size measured for HDLp-A is consistent with that reported for HDLps from other species (17-19). In contrast to the other HDLps, the concomitant association of two molecules of apoLp-III likely contributes to the overall spherelike morphology of HDLp-A.

Based on these results it is conceivable that the neutral lipid deficient, apolipoprotein/phospholipid complex referred to as VHDLp-E possesses the structural capacity to accept and stabilize increasing amounts of neutral lipids. From the observed morphologies of the different subspecies examined it seems most likely that exogenous lipid occupies a central pocket and is surrounded by the apolipoprotein/phospholipid matrix which can adapt its structure to accommodate varying amounts of neutral lipid. When the maximum amount of neutral lipid is reached, a spherical structure is achieved. This is consistent with the fact that additional neutral lipid can be added to HDLp-A only when there is concomitant binding of up to 16 molecules of apoLp-III (16, 20).

Circular Dichroism Spectroscopy CD experiments were conducted in order to assess whether the morphological differences observed in lipophorin structure as the neutral lipid content changes are accompanied by changes in apolipoprotein secondary structure. Spectra were obtained for HDLp-W1, HDLp-W2 and VHDLp-E (Figure 2) but not for HDLp-A because, in addition to apoLp-I and apoLp-II, it contains two molecules of apoLp-III. The CD spectra for the lipophorins exhibited a minimum at 218 nm for HDLp-W1 and HDLp-W2 and at 220 for VHDLp-E, whereas the maxima occurred at 193 nm for both HDLp-W1 and HDLp-W2 and at 194 nm for VHDLp-E. The positions of the minima and maxima are suggestive of the presence of a β-like structure for these proteins which is, in turn, reflected in the CONTIN analysis of the CD spectra (Table 5.1). While it is appreciated that these analyses are based on the CD spectra of globular proteins, and may not be entirely applicable to lipoproteins, the minor variations in the relative amounts of the various structural forms among the three lipophoprins implies that only minor changes in the global protein structure occur as lipid is added or removed. In particular, HDLp-W1 and HDLp-W2 comprise essentially identical amounts of the three conformers, whereas VHDLp-E has a comparable amount of α-helix but a reduction in ß-structure with a corresponding increase in random coil.

Proteolytic digestion. It has been shown previously that apoLp-I and apoLp-II of *M. sexta* larval lipophorin display a differential susceptibility toward proteolysis by trypsin (21). Generally, apoLp-I has been observed to be proteolyzed to a much larger extent than apoLp-II in limited proteolysis experiments (22,23). This result, along with immunological and radiolabeling evidence, has led to the suggestion that apoLp-II may not be exposed to the aqueous environment to the same extent as apoLp-I (24,25). As an extension of this experimental approach we tested different lipophorin subspecies for apoprotein susceptibility to trypsin digestion. The objective of these experiments was to determine the effect of particle neutral lipid content on the degree of apolipophorin susceptibility to proteolysis. HDLp-A was not included in these experiments because it contains apoLp-III, which could also affect the accessibility of apoLp-I and apoLp-II to the enzyme. The results, shown in Figure 3, revealed that, under the conditions employed, the apolipoproteins in HDLp-W1 were relatively resistant to proteolysis, with only a small amount of breakdown of apoLp-I during the course of the experiment. By contrast, the HDLp-W2 and VHDLp-E apolipoproteins showed an increasing susceptibility to proteolysis. In both these particles apoLp-I was preferentially degraded to lower molecular weight components, while apoLp-II was relatively more resistant. When the amount of trypsin was increased to a 1:1 ratio, each of the substrate lipoproteins was degraded to low molecular weight products (M_r < 35,000), indicating that HDLp-W1 is not inherently trypsin insensitive (data not shown). Taken together the results suggest that increasing the neutral lipid content of lipophorin particles confers apolipoprotein resistance to proteolysis by preventing the protease access to basic residues.

Fluorescence studies. The relative exposure of tryptophan residues to fluorescence quenching by acrylamide was determined in different lipophorin subspecies. The fluorescence emission maxima were 325 nm for HDLp-W1 and 327 for HDLp-W2 and VHDLp-E. Based on the tryptophan fluorescence maxima observed for fully solvent exposed apolipophorin I (351 nm; 26) these values indicate the presence of buried or partially buried tryptophans. It is known that 32 of the 34 tryptophan residues present in lipophorin are located in apoLp-I (24). Due to the large number of tryptophans in the molecule an averaging effect will be noted. Figure 4 shows Stern-Volmer plots for HDLp-W1, HDLp-W2 and VHDLp-E. Clear differences were observed in the accessibility of tryptophan residues present in the lipophorin subspecies to quenching by acrylamide, with increasing quenching observed as the lipid content of the particles decreases. This is substantiated by the increasing K_{SV} values, derived from the slopes of the plots in Fig. 4, which yielded values of 1.254 M^{-1}, 1.558 M^{-1} and 2.744 M^{-1} for HDLp-W1, HDLp-W2 and VHDLp-E, respectively (27,28). A reasonable interpretation of these data would be that interaction of tryptophan residues with lipid in HDLp-W1 shields these residues from the quencher, whereas as the lipid content is depleted the residues become more exposed to the solvent.

Discussion

One of the most intriguing aspects of the multifunctional transport lipoprotein, lipophorin, is its capacity to function as a reusable lipid shuttle (17). It is possible that hemolymph LTP (1) plays an important role in lipophorin interconversions by mediating changes in the lipid content of lipophorin subspecies via facilitated net lipid transfer. It is generally accepted that lipophorin meets the bulk of physiological lipid transport demands without destruction of its basic matrix structure; for example, Downer and Chino (29) found that whereas DG has a half life of 2-3 h in hemolymph, apoLp-I and -II have a half life of 5-6 days. Other studies have demonstrated that lipophorin protein components are conserved while functioning to transport DG from fat body storage depot to tissues of utilization (30). Furthermore it has been shown that specific lipophorin subspecies are interconvertible and that naturally occurring, developmentally regulated subspecies of lipophorin arise from modification of the lipid content and composition of preexisting particles rather than from new lipophorin (or apolipophorin) biosynthesis (2). Since many of the changes in the lipid content of lipophorin subspecies occur without addition or removal of apolipoprotein, it is of interest to examine the effect of varying lipid content on the structural properties of lipophorin. While it is generally accepted that HDLp-A is a spherical particle, the effect of DG depletion on its structural characteristics has not been examined in detail. It could be that as the neutral lipid content of lipophorin decreases, other components, such as apoLp-II, reorient to occupy a greater portion of the particle core with maintenance of an overall spherical structure. Alternatively, loss of neutral lipid from the core of the particle could generate nonspherical entities.

The data reported in the present paper support the latter alternative and has important implications with respect to the development of structural models for lipophorin. In subspecies other then HDLp-A a spherical structure cannot be assumed; in fact as the density of the subspecies increases (and lipid content decreases) there is a progressive deviation from a spherical structure. The observed changes in morphology have been correlated with changes in apolipoprotein susceptibility to proteolysis as well as with the relative exposure of tryptophan residues to fluorescence quenching by acrylamide. If the lipophorin particle maintained a spherical structure as it lost neutral lipid, an increasing proportion of apoLp-I and/or apoLp-II would be required to replace lipid in the core of the particle. This would result in a particle of smaller diameter but with an overall decrease in the amount of apolipoprotein exposed to the aqueous environment. Contrary to this prediction we observed a dramatic increase in apolipoprotein susceptibility to proteolysis and tryptophan fluorescence quenching as the neutral lipid content of the particle was decreased. These results support the conclusions drawn from the electron micrographs which suggest that an apolipoprotein/ phospholipid framework encloses a central cavity to which lipid can be added or from which it can be removed. This structural arrangement could conceivably be designed to stabilize the lipid depleted basic matrix structure or to expose cryptic membrane docking or receptor sites (31,32) so that intracellular lipid stores can be accessed.

The results of CD spectroscopy on HDLp-W1 and HDLp-W2 are in general agreement with results previously reported for lipophorins (23,33) although the CONTIN analysis suggests the proteins contain 10-15 % less β-structure. VHDLp-E contained a decreased amount of β-structure and an increase in random coil compared to HDLp-W2 and HDLp-W1 but had a similar amount of α-helix. The increase in random coil at the expense of β-structure together with its low content of neutral lipid and asymmetric morphology, suggests the overall secondary structure of lipophorin is influenced by its lipid component. The relatively small changes in CD indicate that the framework does not reorient to any major extent and suggest increases in proteolytic susceptibility and tryptophan fluorescence quenching reflect the exposure of protein caused by emptying the interior cavity of neutral lipid.

In a study of the transformation of HDLp-A to VHDLp-E in *M. sexta* oocytes, Kawooya, Osir and Law reported that VHDLp-E has a smaller size and elutes later than HDLp-A when subjected to gel permeation chromatography (7). This conclusion assumes that both particles are spherical. However, the electron micrographs of VHDLp-E show an asymmetric object with obvious structural flexibility. When taken together with its ability to reversibly bind lipid (17), the data suggest VHDLp-E should not be viewed as a rigid structure but rather as an apolipoprotein/phospholipid framework that exhibits orientational freedom compared to relatively lipid enriched lipophorin subspecies. This is likely due to the fact that VHDLp-E generally lacks neutral core lipids which require sequestration from the aqueous environment thereby imposing restrictions on the apolipoprotein/phospholipid framework flexibility. The present results regarding the morphology of VHDLp-E are not in agreement with an earlier report by Chino *et al.* (34) who studied hemolymph and egg lipophorins of the silkworm *Philosamia cynthia.* These investigators reported that HDLp-A and VHDLp-E displayed essentially the same morphology, although VHDLp-E was largely depleted of lipid. They offered no explanation as to why these two particles would have the same diameter in spite of their substantial differences in lipid content. In contrast, we have shown a dramatic change in lipophorin morphology consistent with differences in lipid composition.

In a study of larval lipophorin of *M. sexta* (density = 1.15 g/ml), which contains the same two apolipoproteins and an overall lipid content intermediate between HDLp-W1 and HDLp-W2, Pattnaik *et al.* also performed electron microscopy (21). Although these workers did not differentiate among different subspecies micrographs of their preparation exhibited significant heterogeneity and contained many objects which resemble our HDLp-W1 and HDLp-W2 particles.

The morphologies of HDLp-W2 and VHDLp-E are different from that of mammalian lipoproteins, which are spherical in all classes ranging from high density lipoprotein to chylomicrons. Lipoproteins found in the hemolymph of Crustacea such as the spiny lobster, *Panulirus interruptus* (35) or the crab, *Cancer antennarius* (36), however, are polymorphic. Some particles are similar in appearance to HDLp-W2 or VHDLp-E. Crustacean high density lipoprotein particles are similar to the DG-poor lipophorin subspecies in that they are relatively protein and phospholipid rich and the protein components are of high molecular weight (84,000 - 185,000 daltons). The significance of this observation with respect to lipoprotein evolution has yet to be assessed, but it is tempting to speculate that specialized physiological lipid transport needs of insects that arose during evolution (*i.e.* flight) may have resulted in an adaptation whereby circulating lipoproteins could be modified to accept more neutral lipid resulting in formation of more sphere-like structures. The advantage of such a system is that it permits lipid mobilization without new lipoprotein biosynthesis and results in an increased speed and efficiency of lipid transport processes. In the next chapter we are going to measure the monolayer phospholipid mobility of those lipophorins by ^{31}P-NMR. Later in Chapter 8, we will examine how these lipophorins deal with *in situ* induction of extra DG by phospholipase-C at the expense of phospholipids. The amount of DG dictates the binding of apoLp-III to lipophorin particle.

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Table 5.1

CONTIN analysis of the CD spectra of lipophorins

	percent structural form		
	α-helix	β-form	Random coil
HDLp-W1	34	47	15
HDLp-W2	36	49	15
VHDLp-E	34	35	31

Figure 5.1. [illegible]ctron Micrographs of lipophorin subspecies. A) VHDLp-E; B) HDLp-W2; C) HDLp-W1; D) HDLp-A. Samples (20 µg protein/ml) were negatively stained with 2 % sodium phosphotungstate. Drawings, representative of the morphologies present in the respective micrographs, are shown in the lower right hand corner of each panel. It should be noted that VHDLp-E and HDLp-W2 display a degree of structural flexibility, and the drawings represent an "average" view.

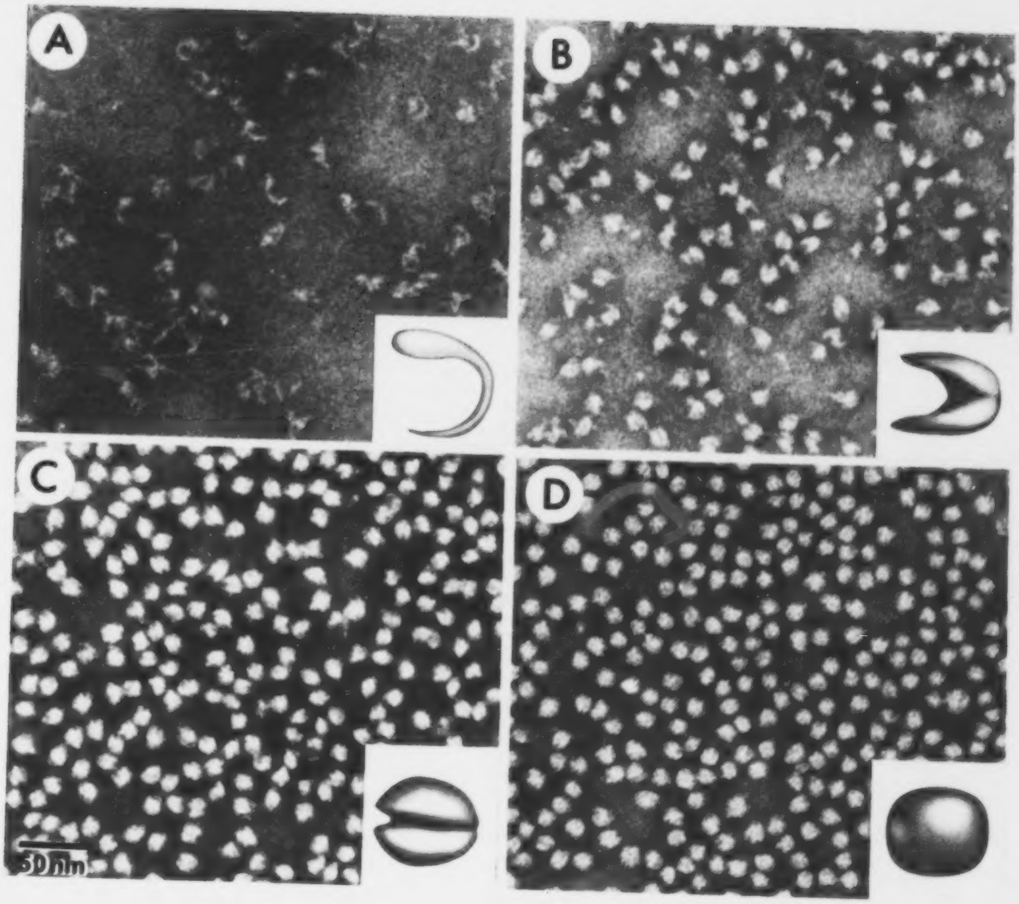


Figure 5.1

Figure 5.2. Circular dichroism spectra of intact lipophorin subspecies. Solid line, HDLp-W1; closed squares, HDLp-W2 and broken line, VHDLp-E. Measurements were taken at 25 °C in 50 mM sodium phosphate, pH 7.0.

Fig 5.2

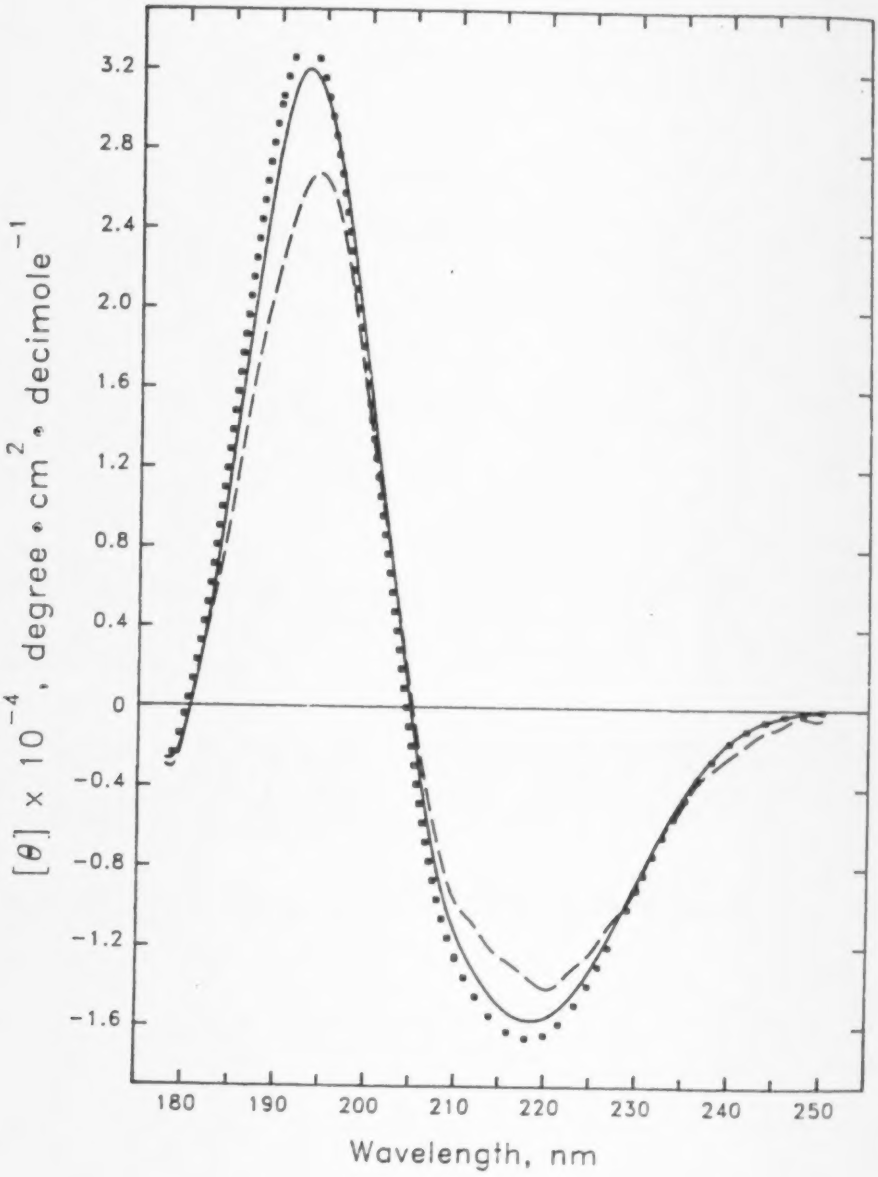

3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0
−0.4
−0.8
−1.2
−1.6
[θ] × 10⁻⁴, degree • cm² • decimole⁻¹
180
190
200
210
220
230
240
250
Wavelength, nm

Figure 5.3. Effect of trypsin on the electrophoretic pattern of lipophorin apolipoproteins. Lipophorin samples were incubated with trypsin (1:200 weight ratio) for various time at 25 °C. After incubation SDS-PAGE sample treatment buffer was added and the samples were placed in a boiling water bath for 2 min. The samples were then electrophoresed on a 4 -15 % acrylamide gradient SDS slab gel. Lanes 1-4, HDLp-W1, control, 10 min, 30 min and 60 min respectively. Lanes 5 -8, HDLp-W2, control, 10 min, 30 min, and 60 min, respectively. Lanes 9-12, VHDLp-E, control, 10 min, 30 min and 60 min, respectively. Lane 13, molecular weight markers.

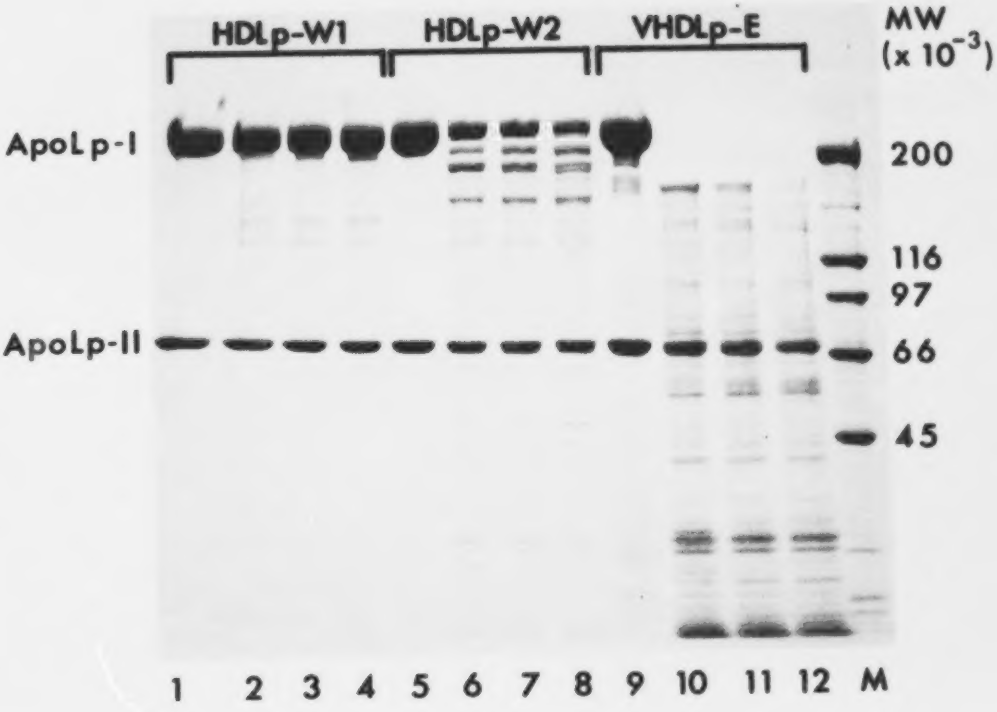
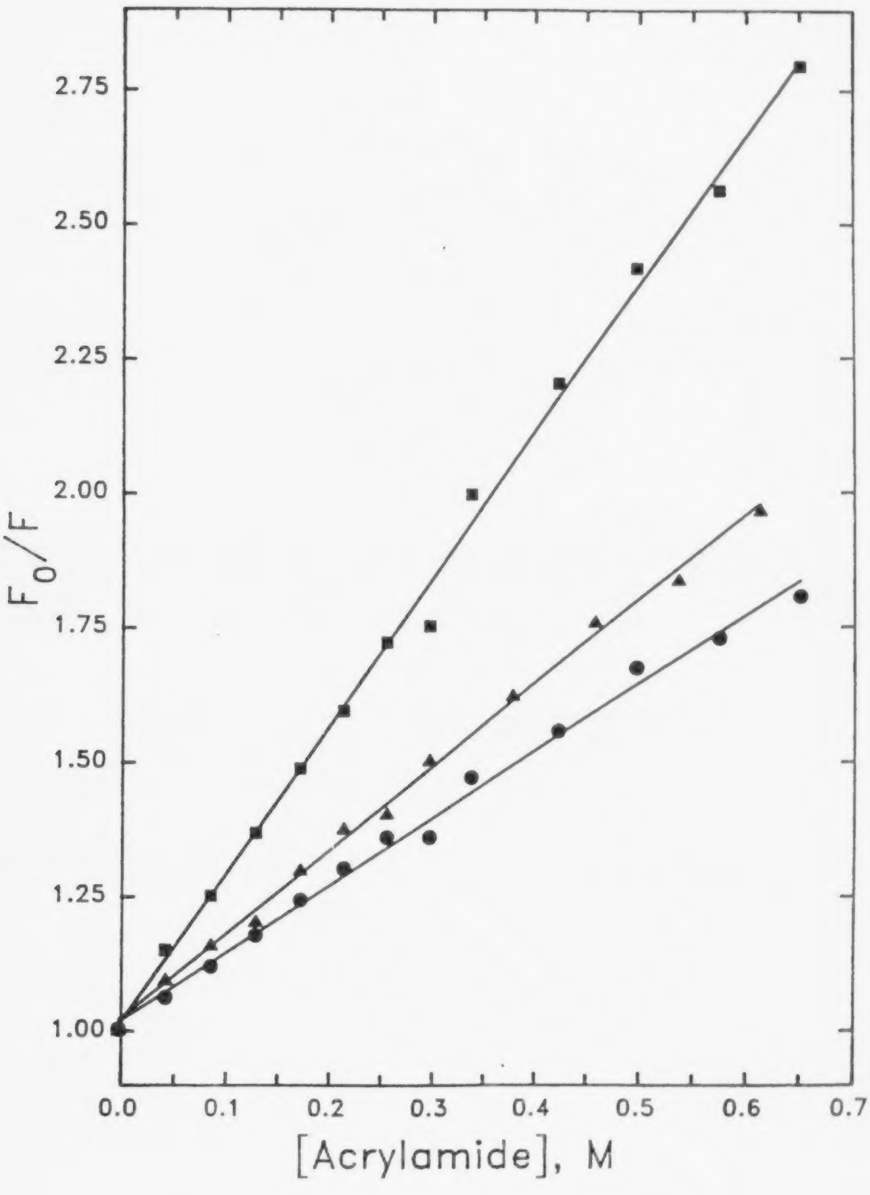

HDLp-W1
HDLp-W2
VHDLp-E
MW
(x 10^{-3})
ApoLp-I
ApoLp-II
200
116
97
66
45
1
2
3
4
5
6
7
8
9
10
11
12
M

Figure 5.3

Figure 5.4. Stern-Volmer plots of acrylamide fluorescence quenching of lipophorin subspecies. Closed squares, VHDLp-E; closed triangles, HDLp-W2 and closed circles, HDLp-W1. Solutions of lipophorin in PBS were excited at 295 nm and fluorescence emission was monitored at 325 nm (HDLp-W1) or 327 nm (HDLp-W2 and VHDLp-E). The concentration of acrylamide in the samples was increased by the addition of aliquots of 8 M acrylamide.

Fig. 5.4


2.75
2.50
2.25
2.00
1.75
1.50
1.25
1.00
F_0/F
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
[Acrylamide], M

CHAPTER 6

^{31}P-NMR Study of the Phospholipid Moiety of Lipophorin Subspecies

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Introduction

The following three chapters will focus on the structure, mobility and biological role of phospholipids in lipoprotein particles.

In insect hemolymph hydrophobic biomolecules are transported via lipid-protein complexes termed lipophorins (Shapiro *et al.*, 1988, Ryan, 1990). The properties of lipophorin have been studied in detail and considerable information is available on their size, apolipoprotein content and lipid composition. In the tobacco hornworm, *Manduca sexta*, it has been shown that during development or in response to hormonal stimuli, dramatic alterations occur in the lipid content and composition of lipophorin particles. Since many of these alterations occur by remodelling pre-existing lipophorin particles, it has been hypothesized that there exists a basic apolipoprotein-phospholipid matrix structure that is capable of accepting or donating lipid to form distinct subspecies (see previous chapter). In spite of large variations in particle lipid content, all lipophorin particles possess one molecule each of the integral apolipoprotein components, apolipophorin I and apolipophorin II (Shapiro *et al.*, 1984; Ryan, 1990). This structural resiliency is a cornerstone of the reusable shuttle hypothesis of lipophorin function first proposed by Chino (Chino & Kitazawa, 1981, Chino, 1985).

In contrast to mammalian lipoproteins, diacylglycerol (DG) is the transport form of neutral lipid by lipophorin. Lipophorin subspecies exist which range in DG content from 4 % to > 20 % of the particle mass (Kawooya *et al.*, 1988; Prasad *et al.*, 1986). Further increases in DG content are observed when a third, low molecular weight, water soluble apoprotein, apolipophorin III (apoLp-III), associates with the particle (Kawooya *et al.*, 1984; Wells *et al.*, 1987, Breiter *et al.*, 1991). High density lipophorin-adult (HDLp-A) contains 25

% DG and two nonexchangeble apoLp-III while low density lipophorin (LDLp), contains up to 16 molecules of apoLp-III and has a DG content of 46 % by weight (Ryan *et al.*, 1986; Wells *et al.*, 1987). The significance of these differences in DG content with respect to the structure or function of lipophorins has not been elucidated. It has been hypothesized that, owing to its relative polarity, DG can partition between the surface and the core of lipophorin particles and, as such, be accessible to transfer from the particle (Ryan *et al.*, 1988).

The other major lipid component of lipophorin is phospholipid which comprises between 14 and 25 % of the mass of different high density lipophorin (HDLp) subspecies (Shapiro *et al.*, 1988). In ^{31}P-NMR and phospholipase A_2 digestion studies, Katagiri (1985) demonstrated that the phospholipid component of *Locusta migratoria* lipophorin is localized in a monolayer on the surface of the particle. This observation is consistent with studies on the location of mammalian lipoprotein phospholipid at the surface of those particles (Keim, 1979). In the present study we have used ^{31}P-NMR to investigate four distinct subspecies of *M. sexta* lipophorin and, based on our analysis, the implications of our results with respect to the structure of lipophorin is discussed.

MATERIALS AND METHODS

Preparation of Lipophorins. Lipophorins were isolated from freshly collected hemolymph of larval or adult stage *M. sexta* by density gradient ultracentrifugation (Shapiro *et al.*, 1984). HDLp-W1 and HDLp-W2 were isolated from prepupal larvae as described by Prasad *et al.* (1986). HDLp-A and LDLp were isolated from one day old adult moths according to Ryan *et al.*

(1986). In some cases, lipophorin samples were concentrated by KBr flotation ultracentrifugation at 1.21 g/ml in a Ti 50.2 rotor at 40,000 rpm for 16 h. The purity of isolated lipophorins was assessed by SDS-PAGE.

Lipid Analysis. Different lipophorin subspecies were extracted with chloroform/methanol according to Bligh and Dyer (1959). Extracts were dried under a stream of N_2 and lipids were separated by thin layer chromatography on glass plates, precoated with silica gel 60, in a solvent system composed of chloroform/ methanol/ acetic acid/ H_2O (50/30/8/3; v/v/v/v). After separation, the bands corresponding to phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were scraped from the plate and subjected to phosphorous analysis (Rouser *et al.*, 1966). Alternatively, the thin layer plates were charred following immersion in 3% cupric acetate (w/v)-8% phosphoric acid (v/v) and heating. After charring, the spots corresponding to PC and PE were quantitated by densitometric scanning on a Cannag TLC Scanner II.

NMR Sample Preparation. Prior to NMR, lipophorins were dialyzed into 30 mM PIPES, 50 mM NaCl, 1 mM Na_2EDTA (pH. 6.8) for 16 h. After dialysis, the samples were used directly for NMR measurements. Using a 10 mm NMR tube, 2.5 ml lipophorin sample was mixed with 0.5 ml D_2O at 4 °C, and placed in the NMR tube. Twenty five microliters of 50 mM inorganic phosphate buffer were added as a reference standard. With the exception of LDLp, lipophorin samples were stable for up to 2 weeks at 4 °C and gave identical ^{31}P-NMR spectra after exposure to 45 °C for several hours. LDLp samples were less stable and thus were used for experiments within 4 days of isolation.

NMR Methods. ^{31}P-NMR experiments were performed on a Varian UNITY 300 NMR spectrometer at 121.4 MHz without proton decoupling. The instrument was equipped with a 300 MHz wide bore magnet for 10 mm NMR tubes. All spectra were taken by using 90° pulse with 2.0 second acqusition time and a relaxation delay of 5-10 s (approximately 5 times T_1). Samples were equilibrated for at least 30 minutes at a given temperature before data were acquired. Spectra were obtained over the range of 0-39 °C with 64-128 transients. The spectral width was 2000 Hz. All spectra were processed by using the line broadening parameter equal to 0.5 Hz.

Spectra of lipophorin subspecies at higher temperatures, such as 25 °C, showed two well separated peaks. As described below, the downfield peak was assigned to PE and the upfield peak to PC. At low temperatures these two peaks were not well resolved. In order to obtain the true linewidth of the peaks at all temperatures, an iterative five-parameter curve fit program (chemical shift of PE, chemical shift of PC, linewidth of PE, linewidth of PC and ratio of intensity of two peaks) was used to fit both PE and PC resonances to double Lorentzian functions. During the curve fit procedure, the intensity ratio of the two peaks, derived from the intensity ratio of the two peaks in different subspecies at 39 °C, was fixed. As described below, the intensity ratio of the two peaks at 39 °C for each lipophorin was in good agreement the PE/PC ratio of the same lipophorin determined by the other methods.

Calculations. The results obtained for intrinsic viscosity, η', and chemical shift anisotropy, $\Delta\sigma$, were based on theoretical considerations (see below) and by using SigmaPlot, version 4.0 (Jandel Scientific, Corte Madera, CA), to curve fit our experimental data. During the curve fit procedure, lipophorin particle radii, determined by electron microscopy (Ryan *et al.*, 1990; Ryan *et al.*, 1992), were fixed. Constraints were also used for intrinsic viscosity (0-10 poise; Edidin, 1974; Marsh, 1988) and chemical shift anisotropy (1-1400 ppm; Fenske *et al.*, 1990).

THEORY

The isotropic motion of the head-group of lipoprotein phospholipids causes ^{31}P-NMR line broadening. This motion arises from two different contributions: whole particle tumbling and phospholipid lateral diffusion within the surface monolayer. Lateral diffusion is a local motion and is much faster than lipoprotein particle tumbling. These two motions modulate ^{31}P-NMR line broadening. The ^{31}P-NMR linewidth of phospholipids in lipoproteins can be written as follows (Abragam, 1961; Mclaughlin *et al.*, 1975; Fenske *et al.*, 1990):

$$1/T_2 = M_2 * \tau_c + C \qquad (1)$$

where M_2 is the residual second moment obtained after averaging due to the restricted anisotropic motion of phospholipids in the plane of the monolayer of the lipoprotein which is given by the following equation (McLaughlin *et al.*, 1975):

$$M_2 = (4/45) * (2\pi\upsilon_0)^2 * (\Delta\sigma)^2 \qquad (2)$$

M_2 is a constant within the temperature range of the present study (Abragam, 1961), C is the portion of the linewidth which is independent of the lipoprotein particle motional correlation time, τ_c (Cullis, 1976) and υ_0 is the ^{31}P absorption frequency.

$$1/\tau_c = (6/a^2) * D_{diff} \qquad (3)$$

where a is the particle radius and D_{diff} is the diffusion rate which consists of two parts:

$$D_{diff} = D_{tumbling} + D_{lateral} \qquad (4)$$

Diffusion arising from whole lipoprotein particle tumbling is given by the Stokes-Einstein relation:

$$D_{tumbling} = kT/ 8\pi a\eta \qquad (5)$$

where η is the viscosity of the medium. If we assume that the intrinsic viscosity, η', of the phospholipid monolayer is homogeneous for each phospholipid class and the phospholipid molecular shape can be described as cylindrical, then the lateral diffusion of phospholipid on the lipoprotein surface can be given as follows (Saffman & Delbruck, 1975):

$$D_{lateral} = (kT/4\pi\eta'\eta)\cdot[\ \log(\eta'\eta/\eta r) - \gamma\ ] \qquad (6)$$

where γ is Eular's constant and r and η are the radius and the length of phospholipid cylinder which can be found elsewhere (Marsh, 1990).

Combining equation (1) with equation (2) , we can obtain:

$$1/T_2 = M_2 * (a^2/ 6D_{diff}) + C \qquad (7)$$

By rearrangement of equation (7) with other equations (4)-(6), the following equation can be derived:

$$(1/T_2 - C)^{-1} = A1 \cdot (T/\eta) + A2 \cdot T + A3 \cdot (T \cdot \log\eta) \qquad (8)$$

where:

$$A1 = 3k/(4\pi a^3 M_2) \qquad (9)$$

$$A2 = 3k\{[\log(\eta'\eta/r)] - \gamma]\}/2\pi M_2 a^2 \eta'\eta \qquad (10)$$

$$A3 = -3k/(2\pi M_2 a^2 \eta'\eta) \qquad (11)$$

Since no phospholipid phase transitions occur in lipophorins over the temperature range 0-37 °C (Katagiri *et al.*, 1985), it is reasonable to assume that η' is independent of temperature within this range. The C term in equation (7) is complex and is attributable to instrumental parameters such as line broadening, acquisition time and phospholipid chemical shift anisotropy resulting from phospholipid interactions with other particle components as well as overall particle structure (McLaughlin *et al.*, 1975). For sonicated egg yolk lecithin bilayer vesicles, C is a constant equal to 15 Hz (Cullis, 1976). The same linewidth for phospholipid resonances within the surface monolyer of lipoproteins, however, is unknown and needs to be determined experimentally. The radius, **a**, of different lipophorins has been estimated from electron microscopic studies (Ryan *et al.*, 1990, Kawooya *et al.*, 1991 and Ryan *et al.*, 1992). Thus, M_2 and η' in equation (8) can be treated as parameters, which can be determined for the PC and PE components of lipophorins as a function of temperature and viscosity, by least-squares fit of equation (8). From the values of M_2 and η', information on the lipophorin phospholipid chemical microenvironment can be obtained.

RESULTS

Assignment of ^{31}P-NMR Signals. The ^{31}P-NMR spectrum of *M. sexta* HDLp-W1 at two different temperatures is shown in Figure 1. The spectrum at 39 °C is characterized by two well resolved resonances at -2.39 ppm and -1.79 ppm while at 3 °C these resonances are less well resolved. By contrast, the ^{31}P-NMR spectrum reported for *Locusta migratoria* high density lipophorin contained only a single resonance which was attributed to PC (Katagiri, 1985). The presence of two distinct resonances in *M. sexta* HDLp-W1 could conceivably be due to the existence of a portion of the phospholipid moiety of this particle in a different microenvironment or the two resonances may arise from different phospholipid classes. These possibilities were examined by obtaining a spectrum of a lipid extract of HDLp-W1 in $CDCl_3$ (data not shown). The spectrum gave rise to two resonances in the same proportion as that found in intact HDLp-W1, indicating that the two resonances observed are not the result of distinctive protein-phospholipid or phospholipid-core lipid interactions but rather appear to be an intrinsic feature of the phospholipid components of this lipophorin. It is known from earlier ^{31}P-NMR studies that certain phospholipid resonances, including those for PE, sphingomyelin and phoshatidylserine are shifted downfield relative to PC due to an ability to form an intramolecular hydrogen bond between the phosphate oxygen and amino or hydroxyl groups within the molecule, which results in deshielding of the phosphorous nucleus (Henderson *et al.*, 1974).

Since previous studies have shown that, in addition to PC, *M. sexta* larval lipophorin contains significant amounts of PE (Pattnaik *et al.*, 1979), we determined the PC/PE phospholipid mass ratio in HDLp-W1 by two independent methods. As shown in Table I, the results correlate well to the integration of the two peaks observed in ^{31}P-NMR spectra of HDLp-W1. When ^{31}P-NMR spectra for other lipophorin subspecies were obtained, they invariably revealed the presence of two resonances, although their ratios were different from those present in HDLp-W1. Mass analysis of the phospholipid component of these subspecies, however, was in good agreement with the observed ratio of the two resonances in each lipophorin subspecies (Table 6.1). Thus we conclude that, of the two resonances observed in ^{31}P NMR spectra of *M. sexta* lipophorins, the upfield peak is due to PC while the downfield peak is due to PE.

^{31}P NMR linewidths of PC and PE in Different Lipophorin Subspecies. The ^{31}P NMR linewidths for PC and PE at 27 °C for each of the four lipophorin subspecies examined in this study are given in Table 6.2. For HDLp-W2, HDLp-W1 and HDLp-A the $\Delta\upsilon_{1/2}$ values for PC and PE were very similar while those for LDLp were broadened by 7-10 Hz. In each subspecies there was a constant 0.61 ± 0.01 ppm chemical shift difference between PC and PE resonances. In all cases plots of chemical shift versus temperature were linear over the range 0-39 °C.

Determination of τ_C Independent Portion of the Linewidth of ^{31}P-NMR Resonances for PC and PE in Different Lipophorin Subspecies. ^{31}P-NMR spectra of HDLp-W1, HDLp-W2, HDLp-A and LDLp were obtained at temperatures ranging from 0-39 °C. Solvent viscosity varied as a function of temperature between 0.5 and 2.4 cP over this temperature range. From the experimental data, we determined the ^{31}P-NMR τ_C-independent portion of the linewidth (C) for both PC and PE in different lipophorin subspecies from plots of $1/T_2$ vs η/T as shown in Figure 2. The calculated values of C are given in Table III. In general the values of C for PC and PE in all lipophorins subspecies were less than C determined by Cullis (1976) for egg PC in sonicated vesicles (15 Hz) and were close to the corresponding linewidth determined for PC and sphingomyelin in human low density lipoprotein (Liu *et al*, unpublished results). The C values obtained for PE compared to PC in the same lipophorin subspecies were consistently larger. For HDLp-W2, HDLp-W1 and HDLp-A, C values for PE were about 2 times greater than that of PC, but for LDLp, C values for PE was only slightly larger than that of PC.

Chemical Shift Anisotropies, Intrinsic Viscosities and Lateral Diffusion Coefficients of PC and PE in Lipophorin Subspecies. From the ^{31}P-NMR linewidth data obtained as a function of temperature and solvent viscosity, the chemical shift anisotropy and intrinsic viscosity of PC and PE in different lipophorin subspecies were calculated. Initial values for both parameters were selected at random and, by fitting the experimental data, the program ultimately arrived at the values reported in Table 6.4. The observation that, in all cases, η' values for PC and PE within the same subspecies are essentially the same suggests the surface of lipophorins can be considered as a single homogeneous enviroment. The data also show that, among HDLp-W2, HDLp-W1 and HDLp-A, intrinsic viscosities are very similiar, but differ considerably from the corresponding values in LDLp, which are about 4 times greater than values for other lipophorin subspecies. The intrinsic viscosity values obtained are within the range reported for a variety of membrane systems (Ladbrooke, *et al.*, 1968; Cone, 1972; Radda & Smith, 1970; Rudy & Gitler, 1972; Grisham & Barnett, 1973). Unlike η', the $\Delta\sigma$ decreases steadily from HDLp-W2 to HDLp-W1 to HDLp-A to LDLp. No significant chemical shift anisotropy differences, however, were found between PC and PE within the same lipophorin particle. D_T values were similar for PC and PE within each subspecies and between HDLp-W2, HDLp-W1 and HDLp-A. However, D_T values for PC and PE in LDLp were appoximately one third of those observed in other subspecies. These data provide support for the concept that the phospholipid components of LDLp exist in a more restricted enviroment than that in other subspecies.

DISCUSSION

^{31}P-NMR has been used to study the structure and dynamics of phospholipids in different subspecies of *M. sexta* lipophorin. Lipophorin particle motion independent linewidths were obtained experimentally for each of four different lipophorin subspecies. *M. sexta* lipophorins are unique compared to other well characterized lipoproteins because they possess relatively large amounts of PE. The observation that lipophorin PE and PC resonances are well resolved permitted quantitation of differences between PC and PE in the same lipophorin subspecies and among different lipophorin subspecies. The data were interpreted on the basis of structural differences between phospholipid head groups in the same lipophorin as well as possible interactions between phospholipids and lipid or protein in different lipophorin subspecies. The content of DG as well as the surface apolipoprotein, apoLp-III, differ in these respective particles and therefore may influence the spectral properties. Toward this end, the structure and motion of PC and PE on the lipophorin particle surface have been characterized by their intrinsic viscosity, η', diffusion coeffcient, D_T and chemical shift anisotropy, $\Delta\sigma$.

τ_C-independent Linewidth of Phospholipids in Lipophorins. The ^{31}P-NMR τ_c-independent portion of PC and PE linewidths (C) for each lipophorin subspecies was obtained from plots of $1/T_2$ vs viscosity/temperature. For each subspecies the temperature range used was 0-39 °C which results in a corresponding solvent viscosity range of 0.5 to 2.5 cP. For all lipophorins studied there was a linear relationship between $1/T_2$ and viscosity/temperature with correlation coefficients of 0.99 or greater. The consistency between our data and that of Cullis (1976) for egg PC in

sonicated vesicles (15 Hz) indicates that C values, obtained by linear curve fitting of our data, are reasonable. However, based on the differences in C among lipophorin subspecies and PC bilayer vesicles, it is clear that C is not a constant and needs to be determined experimentally for each system under study. As discussed in the Theory section, C is affected by instrumental parameters and the chemical shift anisotropy of the phosphate nucleus. While both these contributions are important determinants of C, it should be noted that the instrumental contribution to C for the same phospholipid in different lipophorins, or different phospholipids in same lipophorin, can be eliminated by using identical experimental conditions and processing parameters.

Phospholipid Chemical Shift Differences, η', $\Delta\sigma$ *and* D_T. The observation of two distinct resonances in *M. sexta* lipophorin is different than the results reported by Katagiri (1985) for *L. migratoria* lipophorin, which contains only a single resonance, assigned to PC. From Table I, it is clear that, in addition to PC, *M. sexta* lipophorins contain a considerable amount of PE. Rather than the possibility that a fraction of the phospholipid moiety exists in a unique chemical environment, the appearance of a second resonance in *M. sexta* lipophorins can be ascribed to formation, in PE, of an intramolecular 7-membered hydrogen-bonded ring structure involving the phosphate oxygen and its primary amine proton. By contrast, PC, which lacks the requisite dissociable proton in its head-group, cannot form such a hydrogen-bonded structure. The deshielding effect of this hydrogen bond on the phosphate nucleus of PE causes its ^{31}P-NMR signal to shift downfield by 0.6 ppm compared to that of PC (Henderson *et al.*, 1974). When different subspecies of lipophorin were compared, two resonances, separated by 0.6 ppm, were invariably present. The ratio of intensity of these resonances was reflected in the mass ratio of these two phospholipids providing further support for this interpretation. The linewidths of the PC and PE resonances in the same lipophorin species were comparable but differences existed in the linewidth of PC and PE in different subspecies. While HDLp-W2, HDLp-W1 and HDLp-A were similar, PC and PE linewidths were broadened in LDLp, suggesting a more restrictive phospholipid environment in this species.

Values determined in the present study for chemical shift anisotropy, intrinsic viscosity and lateral diffusion coefficient for PC and PE in lipophorin subspecies are in good agreement with data reported by researchers for phospholipids in the other systems (Marsh, 1988; Edidin, 1974; Seelig, 1978; Seelig *et al.*, 1981; Ghosh, 1988; Scherer & Seelig, 1989) including human plasma lipoproteins (Fenske *et al.*, 1990). For example D_T ranges between 2.3 x 10^{-8} cm^2/sec for high density lipoprotein (HDL) to 1.4 x 10^{-9} cm^2/sec for low density lipoprotein (LDL) whereas lipophorin D_T values obtained in the present study ranged from 4.6 x 10^{-8} cm^2/sec for HDLp-W2 to 1.4 x 10^{-8} cm^2/sec for LDLp. The observation that PC and PE moieties within the same lipophorin subspecies have similar intrinsic viscosity and chemical shift anisotropy values suggests these phopsholipids exist in a homogeneous environment on the particle surface. Furthermore, the increased phospholipid η' in LDLp, compared to other subspecies, supports the concept that apoLp-III and/or DG can induce an alteration in the phopsholipid microenvironment.

Chemical shift anisotropy, $\Delta\sigma$, provides information about the orientational order of phospholipid head groups in the surface monolayer of lipophorin subspecies. Table IV indicates that, within the same subspecies, PC and PE $\Delta\sigma$ values are similar. Compared to other lipoproteins, LDLp has $\Delta\sigma$ values similar to that observed for phospholipids in human LDL and very low density lipoprotein (Fenske *et al.*, 1990). In a like manner the $\Delta\sigma$ of HDLp-A associated phospholipids is similar to that of human HDL_2, while HDLp-W2 is similiar to human HDL_3. Among the different lipophorin subspecies, $\Delta\sigma$ values increase as particle protein weight percent increases. Furthermore, as the overall neutral lipid content of particles decreases, $\Delta\sigma$ increases. Thus it is likely that the observed differences in phopsholipid $\Delta\sigma$ represent a combined effect of phospholipid interactions with protein, other surface lipids and/or core lipid components. This effect, though, may be partially compensated by differences in lipophorin particle size which will affect particle tumbling and phospholipid packing within the surface monolayer.

Lipophorin Structure. Table 6.5 gives the content of DG, PE, PC and apolipophorins -I, -II and -III in different lipophorin subspecies which occur naturally during different life stages of this insect. Examination of these compositions reveals a consistent trend toward increasing DG content from HDLp-W2 to HDLp-W1 to HDLp-A to LDLp. In fact the weight percent DG in HDLp-A is approximately double that in HDLp-W2 while that in LDLp is nearly four times that in HDLp-W2. Interestingly, these dramatic increases in particle DG content are not accompanied by addition of significant quantities of other lipids. LDLp and its direct precursor, HDLp-A, however, do contain an additional apoprotein component, apoLp-III, not found on HDLp-W2 or HDLp-W1 (Ryan, 1990). Differences in $\Delta\upsilon_{1/2}$, η' and D_T between the PC and PE components of HDLp-W2, HDLp-W1, HDLp-A and LDLp noted above (Table 6.2 and Table 6.4) could conceivably arise from increased interaction of PC and PE with DG as its concentration in various subspecies increases. Alternatively, phospholipid head group interaction with apoLp-III may also be important in the observed differences. Either explanation, however, is compatible with the concept that PC and PE in the surface monolayer of LDLp are more restricted in their motion versus their motion in other subspecies.

It is generally accepted that DG occurs naturally within membranes (Nishizuka, 1984). Studies with model bilayer membranes have shown, however, that depending on the type of phospholipid and the amount present, DG can cause a lamellar to hexagonal II phase transition (Epand, 1985; Das and Rand, 1986). By analogy, it is plausible that DG exists in the phospholipid monolayer of lipophorins. Unlike bilayer vesicles, lipophorin-associated DG can partition into the hydrophobic core of the particle where it is largely segregated from the head groups of phospholipids in the surface monolayer. Indeed, it is likely that there is a dynamic equilibrium between DG molecules in the particle core and the monolayer surface which may be influenced by the relative abundance of other surface components (ie. phospholipid and apolipoprotein). Based on compositional analysis of various lipophorin subspecies, HDLp-W2 and HDLp-W1 have an apparent excess of these surface components. Thus it may be that the prevailing surface pressure on these particles forces DG into the core. An important question raised by such an explanation pertains to HDLp-A, which contains two apoLp-III. It may be expected that interaction between phospholipid and apoLp-III would result in the appearance of additional or broadened resonances. Experimentally, however, only one set of relatively narrow resonances was found in the spectra of HDLp-A suggesting that phospholipids might not interact with these two apoLp-III in HDLp-A. Indeed, it has been previously shown that these two apoLp-III are not exchangeable with the other fourteen which bind upon conversion to LDLp (Wells *et al.*, 1987) nor can they be displaced by human apoA-I (Liu *et al.*, 1991). Thus it is reasonable to suggest that apoLp-III in HDLp-A may interact more directly with the particle core and function more like integral apolipoprotein components than exchangeable, water soluble, surface apolipoproteins.

In LDLp, however, the DG content increases by nearly 2 fold from that in HDLp-A (25% to 46 %) and an additional 14 molecules of apoLp-III associate with the particle (Ryan, 1990). Accompanying these compositional changes is an increase in particle diameter from 16 to ~24 nm. The observed increase in LDLp size in the absence of an increase in phospholipid content dictates that expansion of the surface creates a new lipid-water interface. This can be compensated for by a partitioning of DG to the surface monolayer. Owing to its very small head group, however, increases in surface content of DG will cause destabilization of the monolayer. We postulate that gaps created between neighboring phospholipids by intercalation of DG can be stabilized by apoLp-III binding. Indeed the elongated amphipathic α-helical segments of apoLp-III (Brieter *et al.*, 1991) appear well suited to perform this function (Kawooya *et al.*, 1986; Singh *et al.*, 1992). Subsequent interaction between apoLp-III and phospholipids could therefore explain the differences in $\Delta\upsilon_{1/2}$, η' and D_T in LDLp compared to other subspecies. A model illustrating the proposed interaction of phospholipids, DG and apoLp-III on the surface of LDLp is shown in Figure 3. The model depicts an amphipathic α-helical segment of apoLp-III and two PC molecules intercalated by a single DG. Molecular modelling reveals that the hydrophobic face of the amphipathic α-helix of apoLp-III can fit comfortably between phospholipid head groups in the space created by the presence of DG. In such a binding scenario, both the hydrophilic face of the α-helix and the phospholipid head groups remain exposed to aqueous medium, thereby stabilizing the LDLp particle strucutre. This model likely also applies to the case of apoLp-III binding to DG enriched human LDL (Singh *et al.*, 1992) and, possibly, mammalian water soluble apolipoprotein binding to lipoprotein surfaces. It is clear, however, that further studies, probing the interactions of DG, apoLp-III and phospholipid in lipoprotein particles or in model bilayer membrane systems, will be required to reject or confirm this hypothesis. In next two chapters we try to address this question by binding apolipoproteins to the extra hydrophobic surface via enzymatical conversion of lipoprotein particles. ^{13}C-NMR study demonstrated that a surface DG population which differs from the majority of core DG can be detected

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Table 6.1: Ratio of (PE+Sphingomyelin)/PC in Lipophorin Subspecies

	(PE+Sphingomyelin)/PC	
	NMR[a]	inorganic[b]
HDLp-W2	0.74	0.74
HDLp-W1	0.60	0.62
HDLp-A	0.46	0.56
LDLp	0.45	0.48

[a] Data were obtained from the integrated area of the PC and PE resonances at 39 °C. [b] Phosphorus analysis was conducted on the PC, PE, and sphingomyelin components of lipophorin extracts after separation by thin-layer chromatography according to Rouser *et al.* (1966). Since ^{31}P-NMR resonances for PE and sphingomyelin have the same chemical shift valus, their amounts, obtained by phosphorus analysis, have been combined for comparison with the ^{31}P-NMR data.

Table 6.2 ^{31}P-NMR linewidths of PC and PE in M. sexta lipophorin

	line width $\Delta\upsilon_{1/2}$ (Hz)[a]	
subspecies	PC	PE
HDLp-W2	32.1	30.3
HDLp-W1	33.3	29.8
HDLp-A	32.1	32.2
LDLp	43.5	39.6

[a] Data were collected at 27 °C in 30 mM PIPES, pH 6.8, 50 mM NaCl, and 1 mM EDTA buffer, and line widths were determined by double-Lorentzian curve fitting of the experimental data.

Table 6.3 τ_c-Independent portion of ^{31}P-NMR linewidths (C) of the PC and PE components of lipophorin subspecies[a]

	HDLp-W2		HDLp-W1		HDLp-A		LDLp	
	PE	PC	PE	PC	PE	PC	PE	PC
C (Hz)	2.45	1.20	5.57	2.15	5.82	2.44	7.3	7.13

[a] Values were obtained from the data in Figure 2. The *Y* intercept of the data for each subspecies was divided by π to convert $1/T_2$ to hertz

Table 6.4 Chemical shift anisotropy, intrinsic viscosity, and lateral diffusion coefficient values for PC and PE of lipophorin subspecies[a]

	particle radius[b] (nm)	$\Delta\sigma$ (ppm)	η' (P)	D_T (cm^2/s)
HDLp-W2				
PC	6.0	111.0 ± 5.0	0.569	4.61×10^{-8}
PE	6.0	106.0 ± 5.0	0.565	4.63×10^{-8}
HDLp-W1				
PC	7.0	91.0 ± 4.0	0.554	4.70×10^{-8}
PE	7.0	86.0 ± 4.0	0.550	4.73×10^{-8}
HDLp-A				
PC	8.0	74.0 ± 3.0	0.560	4.67×10^{-8}
PC	8.0	72.0 ± 3.0	0.566	4.63×10^{-8}
LDLp				
PC	12.0	43.0 ± 3.0	2.469	1.41×10^{-8}
PC	12.0	42.0 ± 3.0	2.248	1.52×10^{-8}

[a] Data were calculated as described in the text under Theory by curve fitting of the experimental data shown in Figure 6.2.

[b] Assuming spherical particles

Table 6.5 Composition and properties of *M. sexta* lipophorin subspecies

	HDLp-W2[a]	HDLp-W1[a]	HDLp-A[b]	LDLp[b]
molecular weight	550000	650000	786000	1500000
density (g/ml)	1.177	1.128	1.07	1.03
apoLp-I (240 kDa)	1	1	1	1
apoLp-II (85 kDa)	1	1	1	1
apoLp-III (18 kDa)	0	0	2	16
protein (%)	65.2	53.1	48.5	37.6
phospholipids (%)	18.9	23.3	14.0	7.1
DG (%)	12.5	20.2	25.0	46.9
other lipids (%)	3.4	3.4	12.5	8.4
PL (mol/mol of Lp)	134	195	142	137
DG (mol/mol of Lp)	100	193	289	1035
DG/PL	0.75	1.0	2.04	7.66
apoLp-III/PL	0	0	0.014	0.117

[a] From Prasad *et al.* (1986). Molecular weights were estimated from the particle protein and lipid compositions.

[b] From Ryan *et al.* (1986)

FIGURE LEGENDS

Figure 6.1. ^{31}P-NMR spectra of HDLp-W1 at 3 °C and 39 °C. The sample was diaylized against 30 mM PIPES, pH 6.8, 50 mM NaCl, 1 mM EDTA for 16 h prior todata collection. The resonance labeled P_i represents inorganic phosphate internal standard.

Fig. 6.1

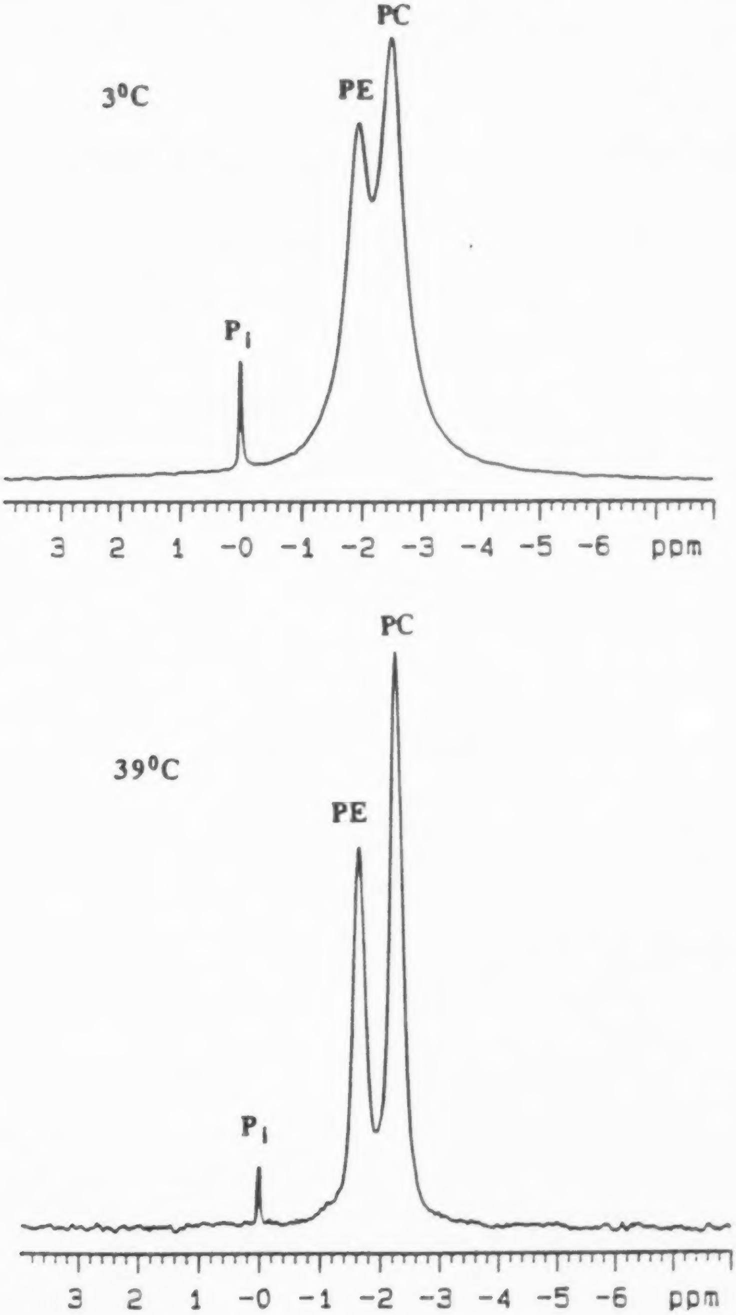


Figure 6.2. Plots of 1/T2 versus η/T for the ^{31}P resonances of HDLp-W2, HDLp-W1, HDLp-A and LDLp. The temperature range was 0-39 °C and viscosity ranged from 0.5 to 2.4 cP. The lines shown were obtained by linear curve fitting of the data (correlation coefficient > 0.99). Open symbols correspond to PC resonances and filled symbols correspond to PE resonances.

Fig. 6.2

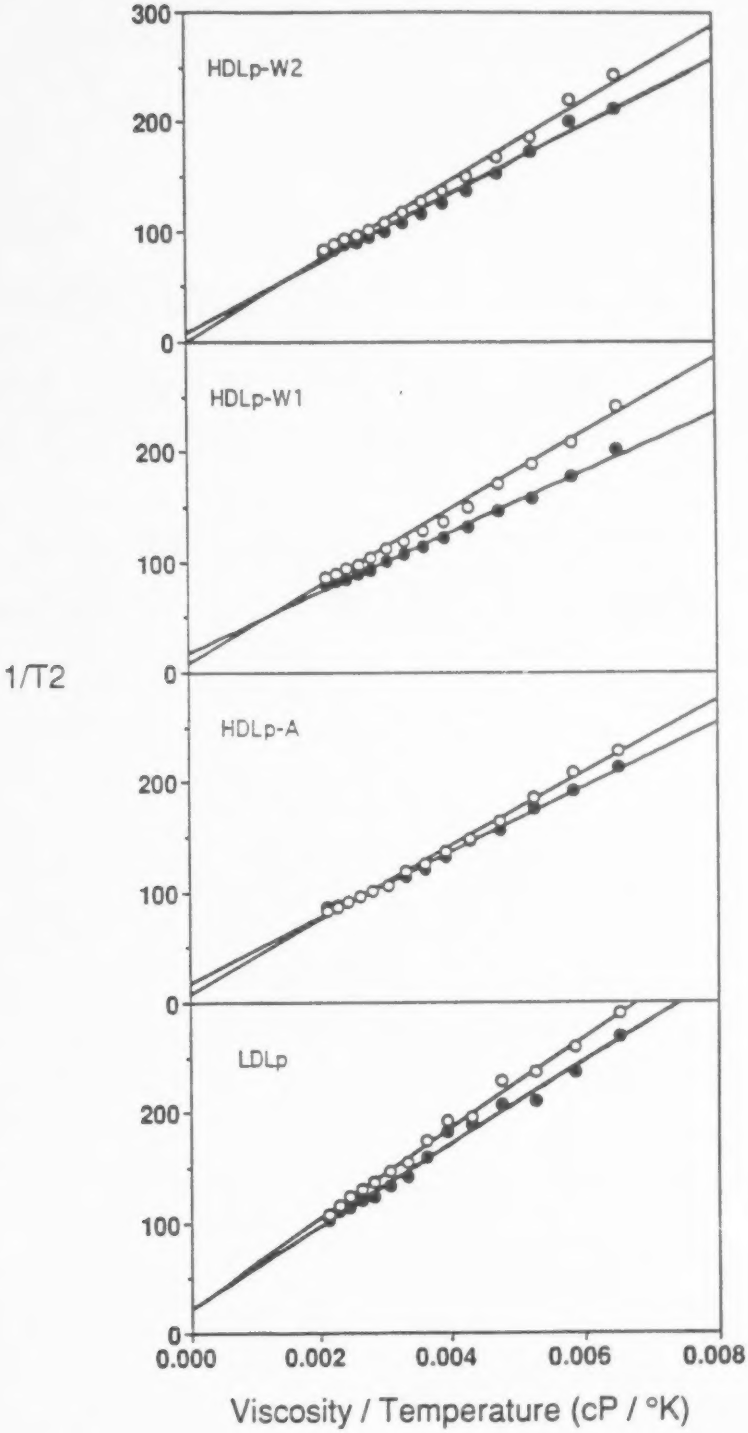
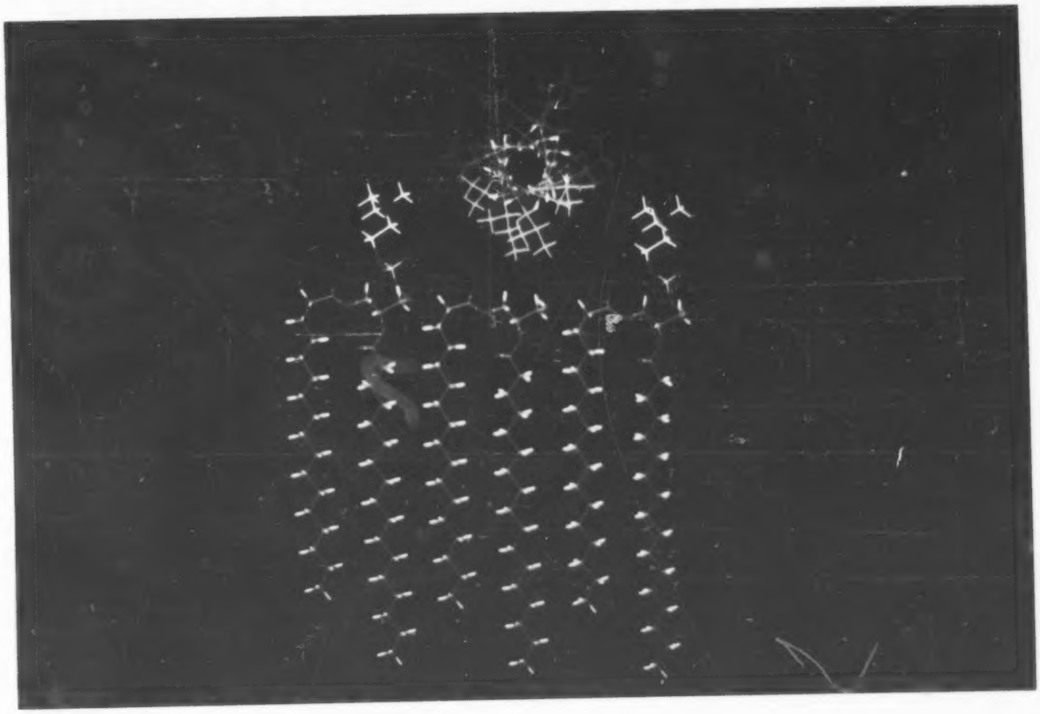

HDLp-W2
HDLp-W1
HDLp-A
LDLp
1/T2
300
200
100
0
0.000
0.002
0.004
0.006
0.008
Viscosity / Temperature (cP / °K)

Figure 6.3. Space filling molecular model depicting an interaction between an α-helical segment of apoLp-III (residues 36 to 45 of *L. migratoria* apoLp-III which is known to be in amphipathic α-helical conformation from X-ray structure analysis; Breiter *et al.*, 1991), with the surface monolayer of LDLp. Two phospholipid molecules, (with the choline head group shown in lavender) intercalated by a single DG, are shown. The hydrophobic face of the amphipathic helix segment (in yellow) is postulated to fill the gap created by the presence of diacylglcyerol. The hydrophilic face of the helix (in red) is oriented toward the aqueous environment.

Fig. 6.3



CHAPTER 7

Prevention of PC-Specific Phospholipase-C Induced LDL Aggregation by Amphipathic Apolipoproteins

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INTRODUCTION

The next two Chapters focus on the effect of enzymatic manipulations of phospholipids on the structural stability of lipoprotein particles.

Low density lipoprotein (LDL) is a metabolic product of very low density lipoprotein (VLDL), and is the major transport vehicle for CEs from liver to extrahepatic tissues (1). LDL and other plasma lipoprotein particles share the common structural structural organization of a hydrophobic core of apolar lipids surrounded by a monomolecular film of amphipathic phospholipids, free cholesterol, and apolipoproteins whose hydrophilic faces project into the water phase (2). Increasing core lipids beyonds its capacity or damage the surface components results in the collapse of particles.

The study of facilitated transfer of DG from insect lipophorin donor particles to human LDL showed that LDL is an excellent acceptor of lipid (3, 4). When the amount of DG transferred exceeds the capacity of LDL to accept it, however, aggregates form (5). Aggregation can be prevented by co-incubation with apoLp-III, a water soluble surface binding apolipoprotein, and in this situation larger VLDL-like particles are formed. In these particles binding of amphipathic apolipoproteins compensates for the increase in hydrophobic molecules (5). A similar phenomenon that occurs under physiological conditions has been observed for the lipid loading process of the insect, *Manduca sexta*. During flight, the lipoprotein DG content increases by 3 fold, converting the resting form of high density lipophorin (HDLp-A) to a low density lipophorin (LDLp), which possesses an additional 14 molecules of apoLp-III (6). Accompanying these compositional changes is an increase in particle diameter from 16 to 23 nm and a decrease in density from 1.08 to 1.03 g/ml.

Phospholipase-C or sphingomyelinase converts phospholipids into DG or ceramide. When LDL is subjected to treatment by either PL-C (8) or sphingomyelinase (9) it aggregates. Aggregated LDL are potentially multivalent (*i.e.*, they contain multiple apo B) ligands for the LDL receptor (10). Enhanced uptake of these aggregates by human and mouse macrophages greatly increases the deposition of cholesterol per internalization event and therefore forms foam cells *in vitro*. These data, together with findings of extracellular phospholipase activities (37), suggest the aggregation of LDL may be an important step in development of atherosclerosis.

The scenario of creation of either DG or ceramide on the monolayer surface of human LDL by PL-C or sphingomyelinase resembles that of excess DG loading into human LDL (5) or insect HDLp-A (6). In both examples, binding of amphipathic apolipoprotein is essential to stabilize the lipid enriched particles (11). We therefore designed the following experiments to investigate if amphipathic apolipoproteins can stabilize LDL particles digested by PL-C, thereby conferring in resistance to formation of atherogenic LDL aggregates.

EXPERIMENTAL PROCEDURES

Materials: PC specific phospholipase C from *Bacillus cereus* (Grade I; < 0.05 % sphingomyelinase activity) was purchased from Boehringer Mannheim, ^{3}H-succinimidyl propionate was from Amersham, and Sephadex G-75 was supplied by Pharmacia. All other reagents were obtained from Sigma or Fisher.

Human low density lipoprotein (LDL, density 1.006-1.063 g/ml) and high density lipoprotein-3 (HDL_3, density 1.125-1.21 g/ml) were obtained from fasting human plasma by sequential density gradient ultracentrifugation. LTP was isolated from *Manduca sexta* according to Ryan *et al.* (12). Human apolipoprotein A-I was prepared by LTP-induced HDL_3 transformation reaction (13). *M. sexta* apolipophorin III (apoLp-III) was purified from LDLp according to the method described by Wells *et al.* (14). HDLp-W1 was isolated from prepupal larvae as described by Prasad *et al.* (15) and HDLp-A was isolated from adult moths according to Ryan *et al.* (16). All the lipoprotein preparations were stored in an argon atmosphere at 4°C.

Analytic methods: Protein concentrations were determined with the BCA assay (Pierce Chemical Co.), using bovine serum albumin as a standard. SDS-PAGE was performed in 4-18 % acrylamide gradient slab gels at 30 mA for 3.5 hours. After electrophoresis the proteins were visualized by staining with Coomassie brilliant blue. Lipid analysis was performed using enzymatic kits for choline containing phospholipids, unesterified and esterified cholesterol, and neutral glycerolipids (Boerhinger Mannheim).

Electron microscopy: Electron microscopy was performed in a Philips EM 420 as described previously (12). Samples (40 mg/ml) were negatively stained with 2 % sodium phosphotungstate (pH 7.0) and photographed at magnifications (calibrated) of 13,000 or 55,000. The diameters of lipoprotein particles were measured on 3x enlarged photographic prints.

^{3}H-succinimidyl apoLp-III labeling: Thirty microliters ^{3}H-succinimidyl propionate (1 mCi/ml) in toluene were dried in a tube under nitrogen. Three micrograms of apoLp-III, dissolved in 300 μl 50 mM Tris-HCl (pH 8.5), was added to the dried tube. After agitating the tube in ice for 10 minutes, the reaction was stopped by adding 200 μl 0.1 M glycine (pH 8.5). The reaction mixture was dialyzed against 150 mM NaCl, 50 mM Tris-HCl (pH 8.0) for 72 hr before use.

Turbidity assay: Microtiter plate wells were used as reaction vessels. Each well contained 50 ml LDL (2 mg/ml) in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl and 2 mM Ca^{++}. PL-C, in the presence or absence of apoA-I or apoLp-III, was added to initiate the reaction. Absorbances at 340 nm were recorded on a SLT Labinstruments Microtiter Reader (17) to monitor the development of solution turbidity. In order to quantitate apoLp-III binding as a function of PL-C hydrolysis, ^{3}H-apoLp-III was added to the incubation medium. After different time intervals the reaction was stopped by adding 200 ml 0.1 M EDTA solution and transferring to ice. Alternatively LDL and apoLp-III were incubated with different amounts of phospholipase C at room temperature, and after 45 minutes the reactions were stopped. In order to remove unbound apoLp-III, the reaction solution was adjusted to a density 1.063 g/ml with KBr in a 13 ml Beckman Quick Seal tube and centrifuged at 40,000 rpm for 24 hours in a Beckman 70.1 Ti rotor. After separation the radioactivity and cholesterol contents of LDL were measured.

RESULTS

PL-C induced LDL aggregation: When LDL was incubated with PC-specific phospholipase-C (PL-C), the light scattering of the solution (measured as absorbance at 340 nm) increased in a sigmoidal manner as a function of time (Figure 7.1). The initial lag period suggests that DG accumulation beyond a threshhold is required before sample turbidity is evident (17). In an identical reaction mixture, the absorbance at 340 nm was allowed to reach 1.0 OD unit, and lipids were extracted with methanol/chloroform (1/3) and separated by thin layer chromatography (TLC). TLC scanning analysis indicated that about 50 % of phosphatidylcholine was hydrolyzed compared to control LDL (data not shown). After 6 hour PL-C incubation ($A_{340\ nm}$=1.21), the phosphatidylcholine content of LDL had declined and the content of DG had correspondingly increased (Table 7.1). Since cholesterol content of LDL is not affected by PL-C, the ratios of glycerolipid/cholesterol and phospholipid/cholesterol of PL-C treated and control LDLs were compared. As expected, the ratio of phospholipid to total cholesterol decreased in the presence of PL-C, whereas the ratio of glycerolipids to total cholesterol increased. These changes are indicative of the hydrolysis of phosphatidylcholine in LDL by PL-C.

Electron microscopic examination of LDL treated with PL-C for various times revealed a progressive aggregation of LDL particles. Figure 7.2 shows representative micrographs of LDL during incubation with PL-C. Panel 1 is PL-C treated LDL at time zero. These LDL particles (panel 1) is homogeneous in size with no sign of aggregation. Panels 2 is the sample when $A_{340\ nm}$=0.21. It shows the beginning of aggregation with small strings and clusters of particles. Panel 3 show the intermediate reaction products when $A_{340\ nm}$=0.82. Although there are many normal sized LDL particles, appreciable number of aggregated clusters have formed. As the PL-C catalyzed hydrolysis continues, increasing aggregation of LDL particles to form stacked clusters is observed; also, some individual particles are beginning to fuse. Panel 4 shows that at the end point of the reaction ($A_{340\ nm}$=1.32), most LDL particles are piled up into large, multilayered aggregates, and fusion of individual particles has continued. Relatively few free particles remain.

Prevention of PL-C induced LDL aggregation by apolipoproteins. In the presence of amphipathic surface apolipoproteins, such as human apo A-I or *M. sexta* apoLp-III, the incubation medium containing LDL and PL-C remained clear; i.e., there was no observable turbidity development (Figure 7.3). This suggested that LDL aggregation was prevented under these conditions. Electron microscopy showed that in the absence of apolipoproteins, PL-C treated LDL formed aggregates and fused into larger particles with an diameters up to 60 nm after 5 hour incubation (Figure 7.4B). A 60 nm diameter particle is equal to 20 LDL-sized particles (22 nm in diameter). In the presence of apo A-I, however, PL-C treated LDL showed no gross morphological alteration (compare panels A and C in Figure 7.4). A few larger, presumably fused particles are observed, suggesting that apo A-I can reverse or block aggregation but that the fused particles via hydrophobic interactions is less reversible.

After the reaction was completed the samples were separated by chromatography on a Sephadex G-75 column in order to remove PL-C and unbound apolipoproteins. The LDL containing fractions were then analyzed for composition, and the ratios of glycerolipid/cholesterol and phospholipid/cholesterol were compared with that of native LDL (Table 7.1). The ratio of phospholipid to total cholesterol was decreased in the presence of phospholipase C in comparison with control LDL indicating that hydrolysis of phosphatidylcholine by PL-C was not inhibited by the presence of apo A-I or apoLp-III.

In order to determine if the added apolipoproteins associated with the surface of PL-C treated LDL, re-isolated LDL containing fractions were subjected to SDS-PAGE (Figure 7.5). In the presence of human apo A-I the gel electrophoretic pattern of re-isolated PL-C LDL samples showed not only apo B but also the appearance of apo A-I. This provides evidence that apo A-I and apoLp-III associated with PL-C treated LDL particles with diminished phospholipids.

Relationship between apoLp-III binding and PL-C induced LDL hydrolysis. In order to investigate a correlation between the extent of PL-C induced phosphatidylcholine hydrolysis and apolipoprotein association, ^{3}H-succinimidyl apoLp-III was incubated with LDL and PL-C at room temperature (Figure 7.6). At different intervals an aliquot of the reaction mixture was removed and the reaction was stopped by adding EDTA and transferring to ice. After separation of LDL from PL-C and excess apoLp-III by density gradient ultracentrifugation, LDL containing fractions were analyzed for both cholesterol and radioactivity. The ratio of ^{3}H-succinimidyl apoLp-III to cholesterol increased as a function of time (Figure 7.6), consistent with the increased turbidity of the control incubation which did not contain apoLp-III (data not shown). This indicated that apoLp-III association with LDL is coupled to the hydrolysis of phosphatidylcholine by PL-C. When LDL samples were incubated with PL-C in reaction wells that did not contain apoLp-III for identical time periods with increasing amount of PL-C, the absorbance increase at 340 nm was proportional to the concentration of enzyme (data not shown). In the presence of ^{3}H-succinimidyl apoLp-III, the amount of radioactivity associated with LDL per mg of total cholesterol showed excellent correlation (R=0.98) with the amount of PL-C added (Figure 7.7). These data demonstrated that the binding of apoLp-III to the surface of LDL treated with PL-C is directly related to the hydrolysis of phospholipid.

DISCUSSION

It has been reported that enhanced uptake of aggregated LDL by cultured macrophages can be induced by mechanical disruption (20, 21), phospholipase-C (8) or sphingomyelinase (9). In each of these cases LDL particle aggregation has been correlated with macrophage cholesterol accumulation. Given the fact that both sphingomyelinase and PL-C are present in serum and many tissues, including the arterial wall (22-26), an enhanced uptake of aggregated LDL may play a role in atherogenesis.

Phosphatidylcholine and sphingomyelin are the major phospholipids in LDL particle. PL-C and sphingomyelinase catalyze the hydrolytic removal of a phosphocholine head group from PC and sphingomyelin, respectively. The hydrophobic hydrolysis products are DG and ceramide. With a much smaller and far less polar head group, these entities in the surface monolayer would be expected to destabilize LDL particles. In this regard it has been shown that incorporation of DG into bilayer membranes promotes a lamellar to reverse hexagonal phase transition (27, 28). To avoid disruption of the surface monolayer, the thermodynamically unstable lipid/water interface created by PL-C hydrolysis must be stabilized. In the absence of amphiphilic lipids entropically driven hydrophobic interactions among phosphatidylcholine depleted surfaces causes the LDL particles to aggregate, and even to fuse. Such aggregation and the formation of larger particles result in solution turbidity. Cellular uptake of aggregated and/or fused LDL induced by PL-C treatment would lead to greater apoprotein B degradation by lysosomal proteases and greater stimulation of cellular cholesterol accumulation, since each internalized aggregated/fused particle would contain multiple apo Bs and much more cholesterol than an individual native LDL particle. With more cholesterol delivered per particle, LDL-cholesterol delivery could exceed cellular cholesterol excretion (29) and cellular cholesterol levels could reach the threshold level necessary for acyl-CoA:cholesterol *O*-acyltransferase (ACAT) stimulation (29) sooner and more extensively. Macrophage derived CE deposition leads to foam cell formation, which is the morphological hallmark of the early cellular lesion of atherosclerosis (30).

We have shown that exogenous amphipathic apolipoproteins can prevent the PL-C induced LDL aggregation. A common feature of these water soluble apolipoproteins is that their secondary structure includes several α-helices. The tertiary structures of *Locusta migratoria* apoLp-III (31) and the LDL receptor binding domain of human apoE (32) have been determined by X-ray crystallography, and it was shown that both have elongated amphipathic α-helices linked by short loops. In the lipid-free state, the hydrophobic surfaces of α-helices point inward while their hydrophilic faces are exposed to the aqueous medium. It has been proposed that upon sensing lipoprotein surface hydrophobicity, the helical bundle would open up and the hydrophobic domains bind to the lipid surface. There is, however, no direct experimental evidence for this conformational change, nor has the mode of binding to lipids been determined.

In adult *M. sexta*, the resting form of lipophorin, HDLp-A, contains 25 % DG. During flight the amount of DG, which serves as an energy fuel, increases more than 3 fold and a larger low density lipophorin particle is formed. The average diameter of lipophorin particles increases from 16 nm to 23 nm (19, 33, 34). While the surface phospholipids do not increase, the increase of DG is associated with the binding of an additional 14 apoLp-III molecules. As the capacity of lipophorin core is limited, more and more DG will partition into the surface monolayer of the lipoprotein particle. Those DG molecules which partition into the surface monolayer will intercalate between the phospholipids since DG has same acyl fatty acid chains as that of phospholipids. DG, however, has a much smaller and much less polar head group, and insertion of a DG molecule between phospholipids will generate a hydrophobic gap which needs to be filled, or the monolayer will be disrupted. Molecular modeling shows that the hydrophobic face of an amphipathic α-helix from apoLp-III will fit between phospholipid head groups in the space created by intercalation of DG (19). The hydrophobic residues used to contact each other in the lipid-free state interact with DG molecule(s), whereas hydrophilic residues likely oriented to interact with phospholipid head groups or to project into the water phase (19).

In studies of DG transfer from insect lipophorin into human LDL catalyzed by the insect LTP, the core of LDL particles possess the capacity to accommodate DG molecules without changing its basic structure. If an excess amount of donor DG is transfered to LDL, however, LDL particles aggregate (5). This aggregation process did not occur when apoLp-III was included in the incubation. A larger, DG, apoLp-III enriched LDL particle was isolated after gel filtration (5). This process is similar to the loading large amount of DG into insect lipophorin, HDLp-A, generating LDLp which has a additional 14 molecules of apoLp-III.

A common scenario which emerges from above observations is that the generation of additional hydrophobic DG molecules is associated with the binding of amphipathic apolipoproteins, which maintains a stable lipoprotein structure. PL-C treatment of LDL represents an alternative method to produce the surface DG *in situ*. In the absence of apoLp-III, LDL particles aggregate as the hydrolysis product, DG, is a destabilizing factor when present in the monolayer of LDL owing to its smaller, less polar head, as well as diminished electric charge which repulses the particle moving toward each other. This destabilization effect is overcome by binding hydrophobic portion of water soluble apolipoproteins, which is consistent with the binding mode as suggested by molecular modelling and ^{31}P-NMR data (Chapter 6, 19).

Both neutral and acidic sphingomyelinase activities are found in the arterial wall (22, 23), liver (24) and serum (26), and extracellular forms of phospholipase C have been reported. These findings suggest that LDL aggregation might occur *in vivo*. LDL particles may be modified by either or both these enzyme(s) before or after their entrance into the arterial wall.

Steinbrecher and Lougheed used gel filtration to demonstrate that most LDLs isolated from plaques of diseased human aortic intimas contain a fraction that eluted in the void volume, and the size of this fraction correlated well with the stimulation of cholesterol esterification. Electron micrographs of this larger sized fraction showed the clusters of LDL particles, larger fused particles with many adherent LDL particles as well as lipid droplets were visualized (35). Aggregates have been found when blood monocytes or intimal smooth muscle cells from normal aorta incubated with LDLs from coronary atherosclerotic and diabetic patients, or with Lp(a). The enhanced cellular uptake of the those aggregated LDL has been observed (36). Our study suggests that LDL aggregation promoted by diminishing phospholipid head groups may be prevented by the binding of water soluble amphipathic apolipoproteins, such as apo A-I and/or apo A-IV, which are present in circulation. Thus, the balance between enzymatic activities specific for phospholipid hydrolysis and the availability of free apolipoproteins may be a determining factor leading to foam cell formation and atheroma. It might be worthwhile to study the regulation of activities of these enzymes at transcriptional, translational, and post-translational levels for both healthy and atherosclerotic subjects.

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Table 7.1 Effect of PL-C and apolipoproteins on the composition of LDL

	LDL apoA-I	LDL PL-C	LDL PL-C apoA-I	LDL PL-C apoLp-III
Total CH (mg/ml)	340±30	460±40	450±30	410±40
GL (mg/ml)	30±2	83±6	65±5	78±6
PL (mg/ml)	95±8	60±5	75±6	50±5
Protein (mg/ml)	210±20	230±20	250±20	240±20
PL/CH	0.28	0.13	0.17	0.12
GL/CH	0.09	0.18	0.15	0.19

CH = cholesterol; GL = glycerolipids; PL = phospholipids. After reaction LDL was re-isolated by gel filtration chromatography. Total cholesterol, glycerolipids, phospholipids and protein were analyzed as described in the text.

Figure Legends

Figure 7.1. LDL aggregation caused by phospholipase-C. Two hundred milligrams LDL were incubated at room temperature with 500 milli units PC-specific PL-C in 150 mM NaCl, 50 mM Tris-HCL (pH 7.5), 2 mM Ca^{++}. Absorbance was monitored at 340 nm. Closed circles, PL-C treated LDL; open circles, control LDL.

Fig. 7.1

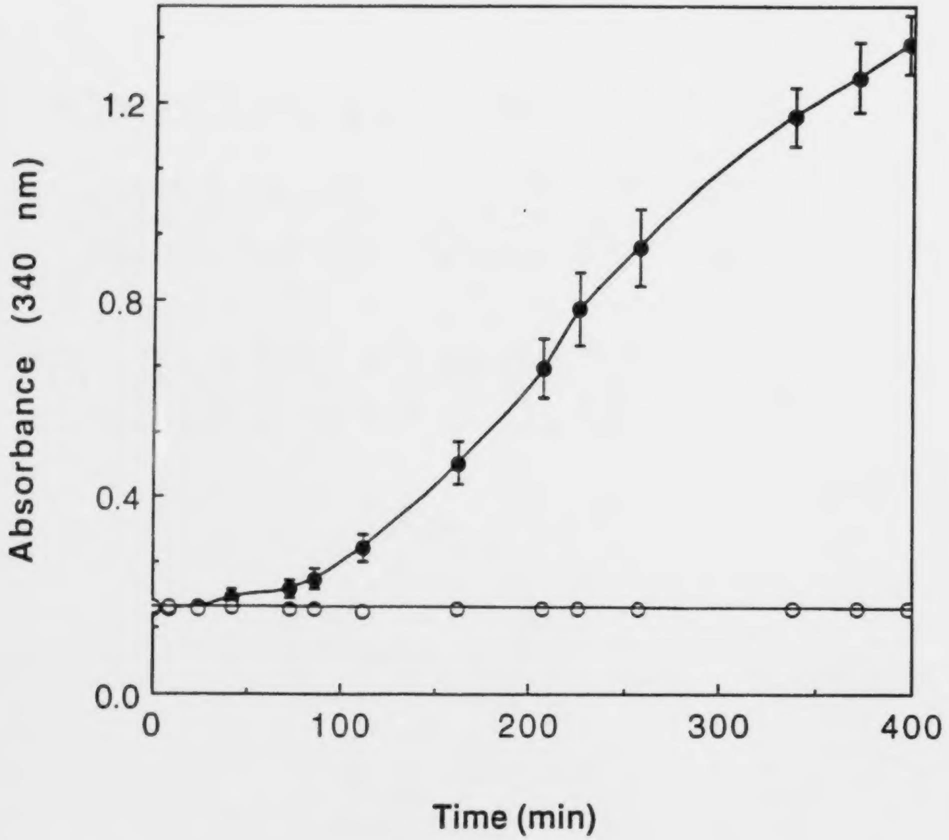


Figure 7.2. Morphological characterization of PL-C treated LDL at different time. Incubation conditions were same as Figure 1. and reaction was carried out at 37 °C. At different intervals, an aliquot of sample was removed and diluted 10 times with 0.1 M EDTA to stop the reaction. Samples were kept on ice until they were negatively stained and photographed in the electron microscope. *Panel a*, before addition of PL-C ($A_{340\ nm}$=0.0); *panel b*, 35 minutes ($A_{340\ nm}$=0.3); *panel c*, 65 minutes ($A_{340\ nm}$=0.7); *panel d*, 285 minutes ($A_{340\ nm}$=1.23).

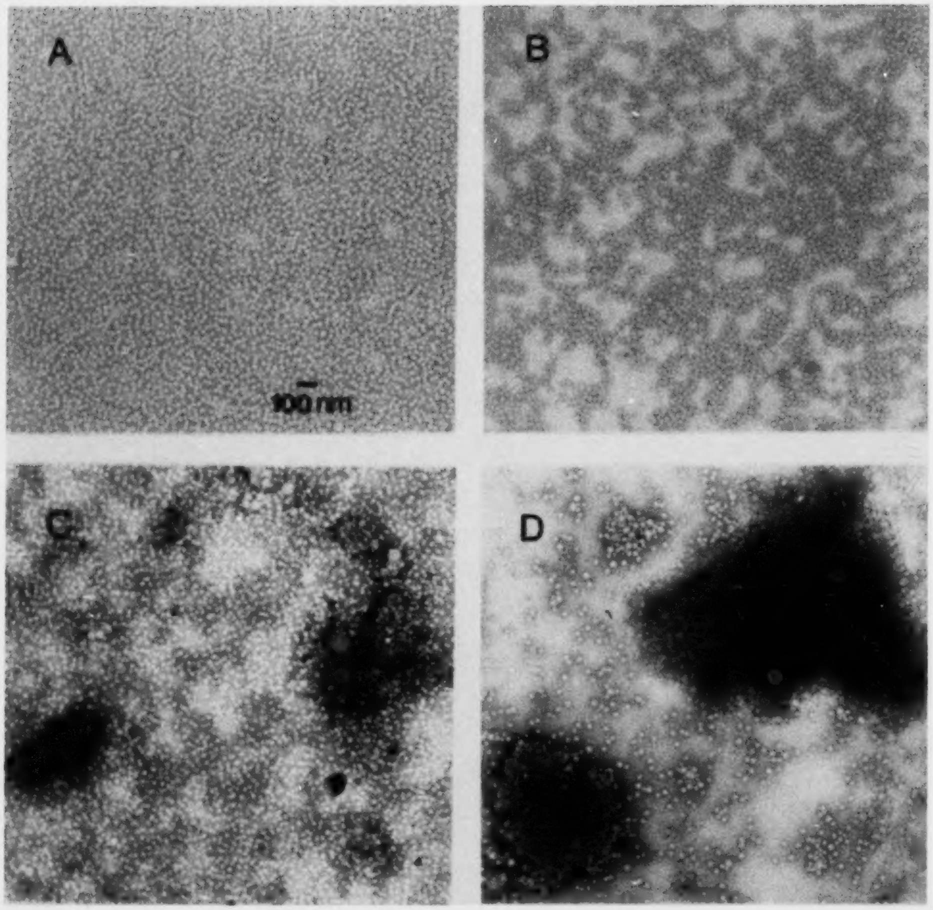


Figure 7.2

Figure 7.3. The effect of polipoprotein addition on the PL-C induced aggregation/fusion of LDL. 200 mg LDL were incubated at room temperature with 500 milli units PC-specific phospholipase-C in the presence or absence of the water soluble apolipoproteins, apoA-I or apoLp-III (200mg each) in same incubation medium as Figure 1.. Absorbances of LDL solutions were monitored at 340 nm. Closed circles, PL-C treated LDL without apolipoproteins; large open circles, untreated LDL; open squares, phospholipase-C treated LDL in the presence of apo A-I; closed diamonds, phospholipase-C treated LDL in the presence of apoLp-III.

Fig. 7.3

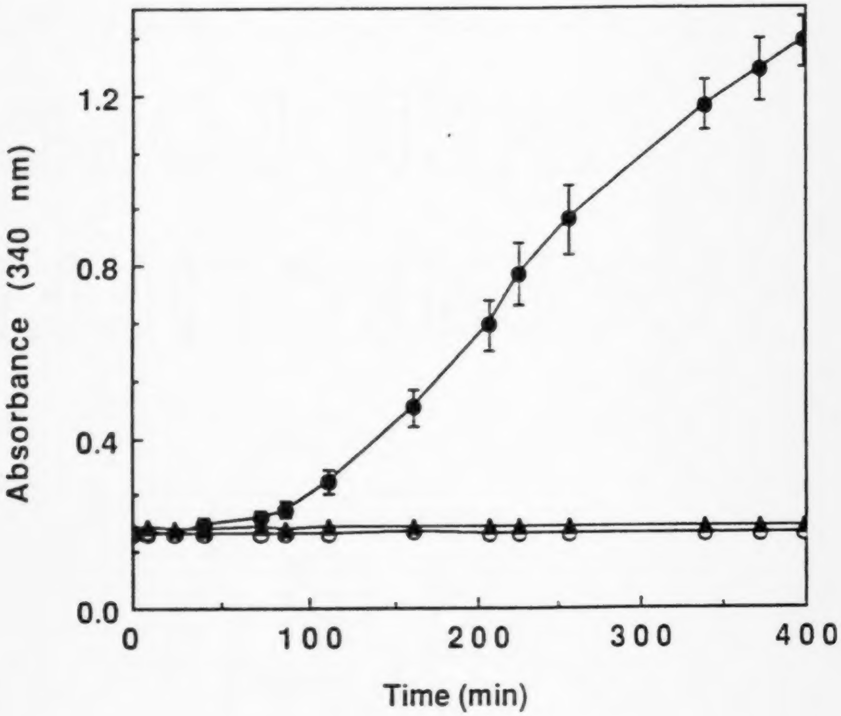


Figure 7.4. Electron micrographs of LDL and PL-C treated LDL in the absence and presence of apolipoprotein A-I. Panel A, untreated LDL particles; panel B, LDL after incubation with PL-C for 285 minutes ($A_{340\ nm}$ =1.23); ; *panel C,* same incubation as *panel B* plus 200 mg apo A-I.

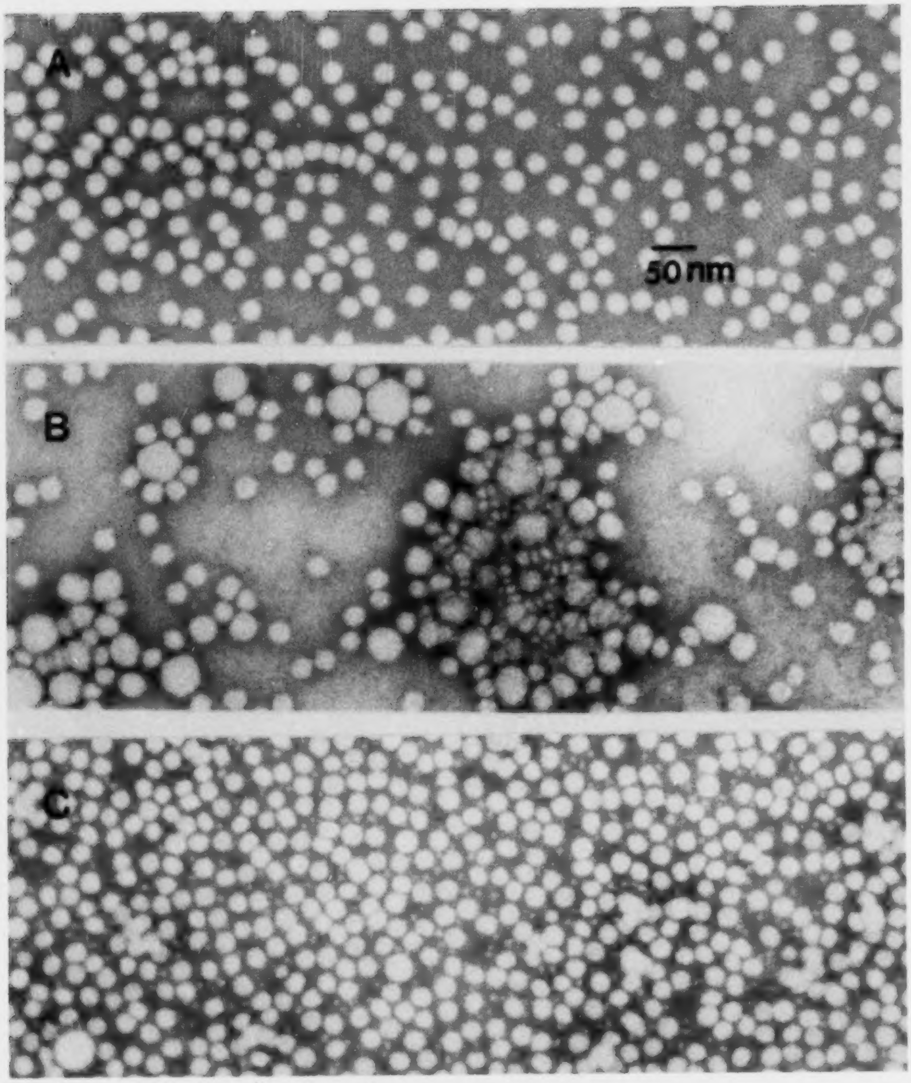


Figure 7.4

Figure 7.5. Apolipoprotein association with PL-C treated LDL. 600 mg of LDL was treated with 1500 milli units of PC-specific PL-C in the presence or absence of an equal amount of apo A-I at 37 °C until the turbidity of a control suspension of LDL incubated without apolipoproteins reached 1.0 at 340 nm. The reactions were stopped by adding 600 ml 0.1 M of EDTA solution, and samples were subjected to gel filtration on a 20x1.5 cm Sephadex G-75 column. LDL containing fractions were applied to a 4-20% polyacrylamide gel and electrophoresed at a constant current of 30 mA for 3.5 h. *lane 1*, molecular markers; *lane 2*, untreated LDL incubation with apo A-I only; *lane 3*, PL-C treated LDL in the presence of apoA-I.

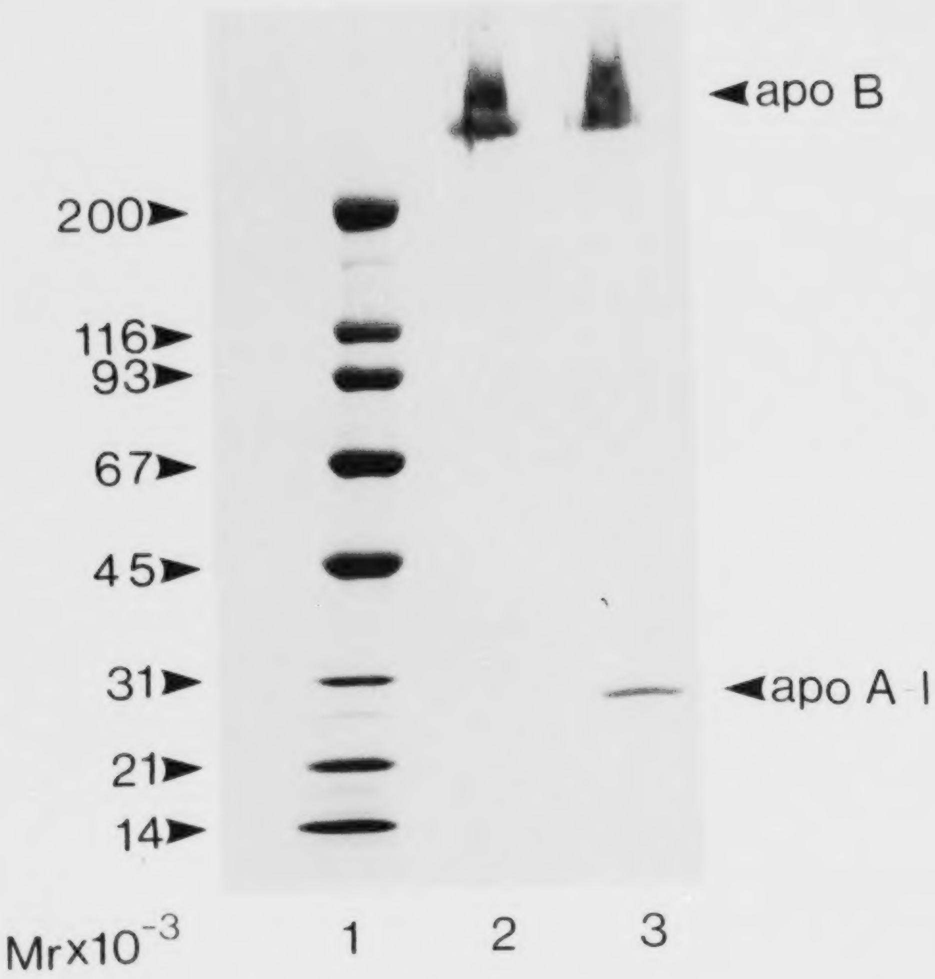


Figure 7.5

Figure 7.6. Effect of the extent of phosphatidylcholine hydrolysis on the association of ^{3}H-succinimidyl apoLp-III with LDL. 400 mg of LDL was treated with 1000 milli units of PC-specific phospholipase-C in the presence of 100 mg of ^{3}H-succinimidyl apoLp-III (1200 cpm/mg) and 300 mg unlabelled apoLp-III. Reaction aliquots were removed at different times. PL-C treated LDLs were separated by density gradient ultracentrifugation, and radioactivity and cholesterol concentration were determined.

Fig. 7.6

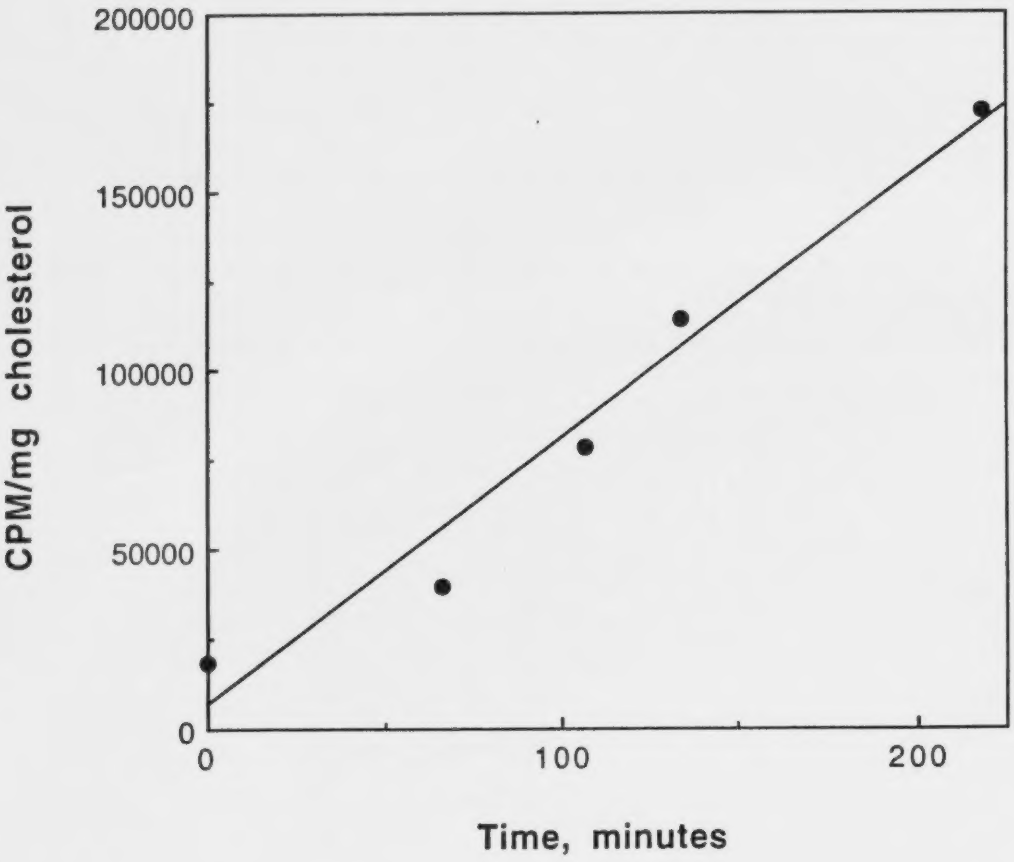
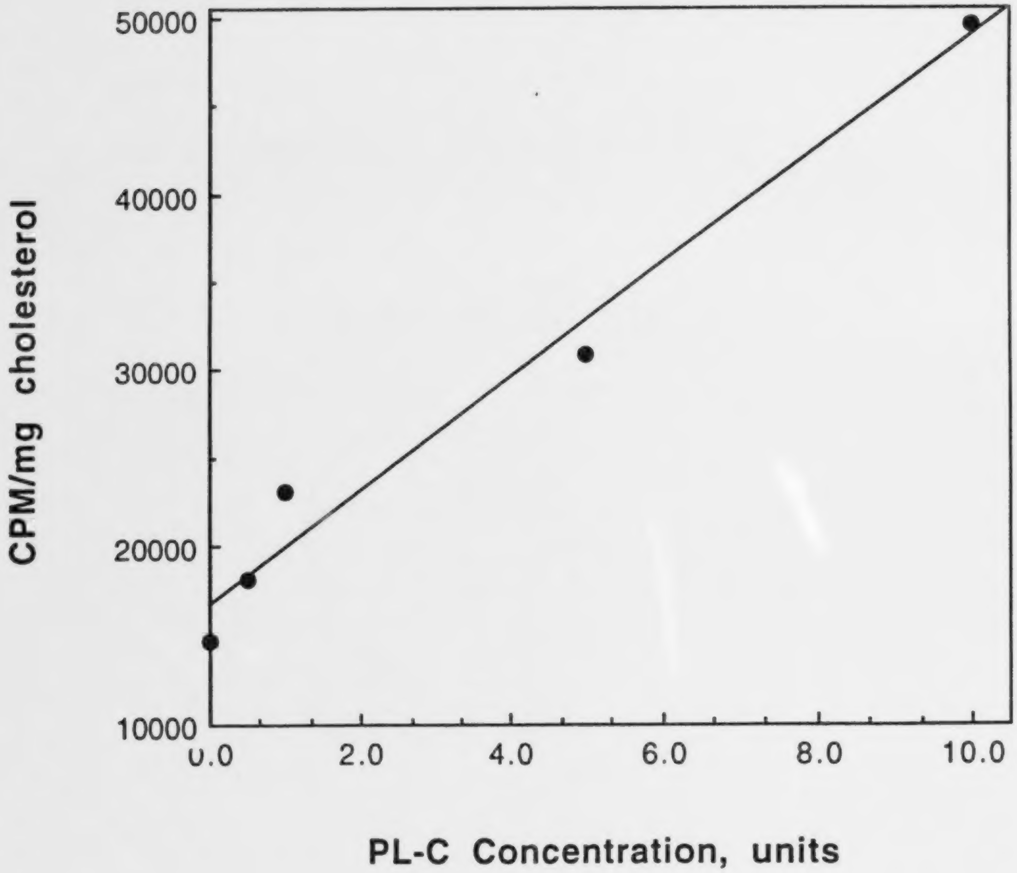

200000
150000
100000
50000
0
CPM/mg cholesterol
0
100
200
Time, minutes

Figure 7.7. Effect of enzyme concentration on the association of ^{3}H-succinimidyl apoLp-III with PL-C treated LDL. LDL and ^{3}H-succinimidyl apoLp-III concentrations were same as for Figure 4, and amounts of phospholipase-C were different as indicated on the X-axis. Reactions were stopped after 40 minutes. Phospholipase-C treated LDLs were then separated, and radioactivity and cholesterol were quantitated.

Fig. 7.7


CPM/mg cholesterol
50000
40000
30000
20000
10000
0.0
2.0
4.0
6.0
8.0
10.0
PL-C Concentration, units

CHAPTER 8

The effect of phospholipase C and apolipophorin III on the structure and stability of lipophorin subspecies

A version of this chapter has been submitted to *J. Lipid Res.* for publication: Hu Liu, T.K. Amareshwar Singh, Roger Bradley and Robert O. Ryan. The effect of phospholipase C and apolipophorin III on the structure and stability of lipophorin subspecies . (1993)

Introduction

In Chapter 5, we found that lipophorins containing different amount of DG in their core have distinct structure and morphology. In this Chapter we will expand our knowledge of how lipophorin structure response to surface phospholipid deletion by phospholipase-C treatment.

In Chapter 7, we demonstrated that human LDL forms aggregates when it is subject to the PL-C phospholipolysis. The PL-C induced LDL aggregation can be prevented by co-incubation with amphipathic apolipoproteins, such as human apo A-I and insect apoLp-III (Liu *et al.*, 1993). As we discussed in the general Introduction, insect lipophorins have some structural characteristics similar to those of human apo B-containing lipoproteins. Together with phospholipids, integral apoLp-I and apoLp-II have similar function as apo B to maintain a stable particle matrix. On the other hand, lipophorins have their unique properties, perhaps the most importantly, containing DG, but not TG and CE, as their major core lipids. Since PL-C hydrolysis converts phospholipids into DG, treatment of lipophorins with PL-C might answer the following interesting questions: (1) what is the effect of these newly induced DG on the structural stability of lipophorin particles; (2) can we differentiate surface from core DG populations. The first question will be the main subject of current Chapter. The next Chapter will try to detect the different locations of DG moiety in the lipophorin particles by ^{13}C-NMR.

In the present study we chose four different lipophorin species which have different morphologies (Ryan *et al.*, 1992) and contain different amounts of DG as their major core lipids, and asked whether these distinct lipoproteins can tolerate the enzymatic creation of DG at the expense of phospholipid. A correlation between the amount of DG generated and the extent of apoLp-III binding has been found which supports the possible binding mode of amphipathic apolipoproteins to the surface of DG enriched lipophorins.

Experimental procedures

Materials. Phospholipase-C from *Bacillus cereus* (Grade I; <0.05% sphingomyelinase activity) was purchased from Boehringer Mannheim, Laval, Quebec, Canada. Larval and adult *Manduca sexta* were obtained from a continuing laboratory colony reared on a wheat germ based diet according to Prasad *et al.* (1986). Lipophorins were isolated from freshly collected hemolymph of larval or adult *M. sexta* by density gradient ultracentrifugation (Shapiro *et al.*, 1984). HDLp-W2 and HDLp-W1 were isolated from prepupal larvae as described by Prasad *et al.* (1986). HDLp-A and LDLp were isolated from 1-day-old adult moths according to Ryan *et al.* (1986). In order to ensure homogeneous lipophorin preparations, samples were centrifuged a second time. ApoLp-III was purified from adult hemolymph as described by Wells *et al.* (1985).

Methods. Protein content was determined with the bicinchoninic acid assay (Pierce Chemical Co.), using bovine serum albumin as standard. SDS-PAGE was performed in 4-20 % acrylamide gradient slab gels and stained with Coomassie brilliant blue. The gel was scanned on a Camag TLC Scanner II. Lipid contents were analysed by enzymatic kits for phospholipids (Wako Chemical Co., Osaka, Japan) and glycerolipids (Boehringer Mannheim). Routine PL-C assays were conducted in microtiter plate wells containing 100 μg lipophorins in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2mM Ca^{++} in the presence and absence of apoLp-

III. PL-C was added to start reaction. Absorbances at 340 nm were recorded on a SLT Labinstruments Microtiter Reader (Singh *et al.*, 1992b) (Liu *et al.*, 1993) to monitor the develoment of solution turbidity. The optimal wavelenth for turbidity detection was determined from difference spectra comparing incubations of each lipophorin in the presence and absence of phospholipase-C. The spectra revealed a similar trend for each subspecies with the maximal absorbance difference occurring between 340 nm and 450 nm.

In some cases lipids were extracted with Chloroform/metyhanol (2:1) and separated by thin layer chromatography using plates coated with silica gel G. Samples were separated in a solvent system containing chloroform/methanol/acetic acid/water (50/30/8/2) and the thin layer plates were charred following immersion in 3% cupric acetate (w/v)-8% phosphoric acid (v/v) and heating. After charring, the spots corresponding to individual lipids were visualized.

Results

Phospholipase C treatment of lipophorin subspecies. The phospholipid moiety of lipophorin is located in a monolayer on the particle surface (Katagiri, 1985). As such they are susceptible to phospholipase C catalyzed hydrolytic removal of their head group resulting in generation of DG. Since a multitude of lipophorin subspecies exist which possess dramatically different lipid compositions (Beenakkers *et al.*, 1988; Wang *et al.*, 1992) we set out an experiment to determine the effect of phospholipase C treatment on the structure and stability of four distinct, well characterized lipophorin subspecies. From studies of human lipoproteins it is known that phospholipase C destabilizes lipoprotein particle structure, inducing particle aggregation

(Chapter 7). Such aggregated lipoprotein samples develop turbidity as the reaction proceeds, permitting the aggregation progress to be monitored spectrophotometrically at 340 nm (Singh *et al.*, 1992b). When equimolar amounts of four different subspecies of lipophorin were incubated with phospholipase C, they displayed a variable extent of solution turbidity development as a function of time (Figure 8.1, panel A). A similar trend was observed when A_{340} was monitored as a function of enzyme concentration (data not shown). LDLp sample turbidity increased to a much greater extent than that of the other subspecies. After three hours under these conditions, sample turbidity reached a plateau suggesting that a reaction end point had been reached. Incubation of LDLp under identical conditions in the absence of phospholipase C had no effect on sample turbidity. HDLp-A and HDLp-W1 samples both displayed much smaller increases in absorbance at 340 nm following phospholipase C treatment. By contrast, phospholipase C treated HDLp-W2 did not develop any increase in sample turbidity after 7 hour incubation, indicating particle aggregation did not occur.

To determine which phospholipids were hydrolyzed, the lipids of four lipophorins treated with and without PL-C were extracted and analyzed by thin-layer chromatography (TLC). Both phosphatidylcholine (PC) and phosphatidylethanolamine (PE) bands in all four PL-C treated lipophorin subspecies were significantly reduced while sphingomyelin was unaffected, compared to the control lipophorins (data not shown). No detection ^{13}C-NMR resonances of phospholipid moiety at carbonyl region after PL-C hydrolysis further confirmed that both PC and PE were converted to DG (see Figures 9.5, 9.6 of next Chapter). In all cases depletion of lipophorin PC and PE content was accompanied by an increase in DG, the expected product of phospholipase C mediated hydrolysis. Since sphingomyelin represents a minor phospholipid component of *M. sexta* lipophorins, these data indicate that nearly all of the phospholipids in lipophorins are substrates for phospholipase C.

The differential solution turbidity of the various lipophorins after PL-C hydrolysis was observed in assays containing same amounts of protein masses. Since the ratio of phospholipid/protein and especially DG/protein differs dramatically among these subspecies, it is necessary to normalize them in same concentration with respect to phospholipid and DG contents. In the assay condition containing same amounts of phospholipid (Figure 8.1, panel B), the lipophorin samples developed a similar trend of solution turbidity as observed in panel A, reflecting that all four lipophorins have similar amounts of phospholipids. When DG content was normalized (Figure 8.1, panel C), LDLp still exhibited the largest turbidity despite the fact this incubation sample contained far fewer particles. HDLp-W2 again failed to develop turbidity even if the assay contained many more HDLp-W2 particles.

The lack of solution turbidity in PL-C treated HDLp-W2 suggests that this lipophorin has an excess of surface components as well as additional core capacity. We have previously shown that HDLp-W2 has an unusual asymmetric morphology (Ryan *et al*, 1992) as well as a composition that is rich in apolipoprotein and phospholipid (Wang *et al.*, 1992). To verify that PL-C hydrolysis did not induce aggregation of HDLp-W2 we examined its morphology by electron microscopy (Figure 8.2). No significantly morphological change was observed between control and PL-C treated HDLp-W2. By contrast, PL-C treated HDLp-W1, which displayed a small increase in sample turbidity, showed clear evidence of particle aggregation. This data supports the interpretation that the observed increase in A_{340} represents a measure of lipophorin particle aggregation.

From studies of human low density lipoprotein it is known that mechanical disruption of lipoproteins causes aggregation and sample turbidity development (Khoo *et al.*, 1988). To determine if the differential extent of aggregation induced by phospholipase C treatment of the various lipophorins was due to inherent structural properties of the particles (e.g. lipid:protein ratio) we subjected the lipophorin samples to mechanical disruption on a "vortex" mixer. In this case the extent of turbidity development revealed a trend similar to that obtained following phospholipase C treatment (Figure 8.3).

Prevention of PL-C induced lipophorin aggregation by apoLp-III.

When apoLp-III, a lipid surface binding, amphipathic α-helical apolipoprotein found in high concentration in insect hemolymph (Wells *et al*, 1987; Breiter *et al*, 1991; Ryan *et al*, 1993), was co-incubated with lipophorins in the presence of phospholipase C, sample turbidity did not develop with any lipophorin (Figure 8.3). An apoLp-III concentration dependent prevention of LDLp sample turbidity development was observed (Figure 8.4). This data suggested that either the enzyme was inhibited by apoLp-III or, alternatively, lipophorin aggregation was prevented by association of apoLp-III with the enzymatically modified lipoprotein surface. In order to differentiate these two possibilities lipid analysis was performed. Table I is the composition of control and PL-C treated lipophorins. In all cases the phospholipase C treatment induced decrease in the ratio of PC to protein was similar in the presence and absence of apoLp-III. Furthermore phospholipase C induced a corresponding increase in the ratio of DG to protein indicating production of DG at the expense of phospholipid. These quantitative results were consistent with results of thin layer chromatography described above and indicate that the ability of apoLp-III to confer resistance to phospholipase C induced aggregation of lipophorin is not due to an inhibition of phospholipase C activity. To confirm that apoLp-III binding resulted in maintaining the particle integrity, in spite of phospholipid depletion, the morphology of LDLp was examined by electron microscopy (Figure 8.5). Control LDLp displayed the expected range of particle sizes (Ryan *et al*, 1990). By contrast, phospholipase C treated LDLp formed large aggregates, consistent with the results of turbidity development. When apoLp-III was present in the incubation of LDLp and PL-C, however, morphologies of LDLp particles are strikingly similar to that of control LDLp. A similar morphological resemblance of PL-C digested HDLp-W1 and HDLp-A were also found when compared with their respective controls (data not shown). Furthermore the formed lipophorin aggregates could not be reversed by adding additional apoLp-III. From these results we hypothesized that apoLp-III-mediated prevention of PL-C induced lipophorin sample aggregation occurs via formation a stable binding interaction.

Binding of apoLp-III to phospholipase C treated lipophorins.

Each of the four lipophorin subspecies were incubated with apoLp-III in the absence and presence of phospholipase C, followed by density gradient ultracentrifugation to remove unbound apoLp-III. Under these conditions lipophorin samples float above lipid-free apoLp-III, which remains in the infranatant. Lipophorin samples reisolated in this manner were subjected to SDS-PAGE (Figure 8.6). Compared to the control, PL-C did not induce the binding of apoLp-III to HDLp-W2, which is consistent with the following observations: 1) phospholipase C fails to induce turbidity in this lipoprotein and 2) PL-C treated HDLp-W2 has similar particle morphology to the control particles. ApoLp-III was found in association with HDLp-W1, however, following phospholipase C treatment. In addition, although apoLp-III is normally found in association with both HDLp-A and LDLp. PL-C treatment of these lipophorins induced additional apoLp-III binding the particles. Using apoLp-II as internal standard, the amount of apolp-III in each lane was estimated by scanning densitometry. Based on the knowledge that native HDLp-A contains 2 apoLp-III and LDLp contains 16 apoLp-III molecules per particle (Wells *et al.*, 1987), we estimate that PL-C treatment induces association of 3-5 more apoLp-III molecules per particle (Table II). The additional apoLp-III molecules apparently compensate for PL-C generated defects in the surface of these lipophorins.

Discussion

Lipophorin subspecies naturally contain different amounts of DG as their major core lipid while maintaining the same integral apolipoprotein components, apoLp-I and apoLp-II (M_r=240,000 and 85,000, respectively; Ryan, 1990). It has been proposed that apoLp-I and apoLp-II, together with phospholipids, comprise a reusable basic matrix structure that is common to all forms of lipophorin. At different life stages this structural framework allows neutral lipids to be loaded onto or removed from lipophorin to meet different metabolic lipid transport requirements (Ryan, 1990; Ryan *et al.*, 1992).

As was the case of phospholipase-C treated human LDL particles (Chapter 7), PL-C digestion of lipophorin results in loss of the polar head groups of both phosphatidylcholine and phosphatidylethanolamine. The product DG, owing to its smaller and far less polar head group, likely destabilizes the monolayer of lipophorin particles. It has been shown that incorporation of small amounts of DG into bilayer membranes promotes a lamellar to reverse hexagonal phase transition (Siegel *et al.*, 1989; De Boeck *et al.*, 1989). To avoid a similar disruption the thermodynamically unstable lipid/water interface on the surface of lipophorins must be stabilized. In the absence of amphiphilic surface lipids, hydrophobic interactions among surfaces on neighboring particles created by PL-C hydrolysis interact to create insoluble aggregates (Liu *et al.*1993).

The observation that different lipophorin subspecies can tolerate phospholipase-C induced loss of phospholipids to different extent (Figure 8.3) suggests that apolipoprotein frame undergoes structural and conformational changes to deal with surface lesions caused by PL-C hydrolysis.

For example, HDLp-W2 contains on average 100 DG and 134 phospholipid molecules per particle (Prasad *et al.*, 1986). Electron microscopy revealed that HDLp-W2 possesses an unusual morphology, containing a central cleft which suggests the particle core is not fully occupied (Ryan *et al*, 1992). Interestingly, the aggregation phenomenon was not observed when HDLp-W2 was treated with PL-C. Since native HDLp-W2 contains relatively little DG the flexible apolipoprotein structural frame can accomodate DG molecules created from phospholipid hydrolysis. Furthermore, since HDLp-W2 possesses an apparent excess of surface components (Wang *et al.*, 1992) association of apoLp-III with the particle surface is not necessary to maintain a stable particle structure. This was confirmed by the facts of no major morphological changes (Figure 8.2) and no observed binding of apoLp-III to PL-C treated HDLp-W2 sample (Figure 8.6, lanes 1 and 2)

HDLp-W1 is metabolically converted from HDLp-W2 through acquisition of more DG (approximately 90 moles/mole lipophorin) and phospholipids (60 moles/mole lipophorin) causing changes in particle morphology toward a more sphere-like structure (Ryan *et al.*, 1992). HDLp-A contains 289 DG and 142 phospholipid molecules, together with 2 molecules of apoLp-III. Compared to HDLp-W2, HDLp-W1 and HDLp-A contain more DG molecules, which occupy nearly the entire core of the apolipoprotein structural frame. A certain amount of the additional DG created via hydrolysis of PC and PE molecules may remain in the surface, forming hydrophobic areas which caused particle aggregation. Although native HDLp-W1 has less DG per particle than HDLp-A, PL-C treated HDLp-W1 displayed more aggregation. This may be due to the fact that HDLp-W1 bears more phospholipid molecules than HDLp-A (195 vs 143 molecules per particle, respectively) or, alternatively, may be attributable to the presence of two apoLp-III on HDLp-A.

During flight, DG is loaded onto HDLp-A forming LDLp particles which display an increase in diameter from 16 nm to 24 nm and a decrease in density from 1.08 to 1.03 g/ml (Ryan *et al.*, 1986; Ryan *et al.*, 1990). Compositonal analysis shows LDLp contains approximately 1035 DG molecules. Compared to HDLp forms, PL-C treatment of the much larger, DG-enriched LDLp induced an even greater extent of particle aggregation.

As we discussed in Chapter 7, reversible loading of DG *in vivo* (Ryan, 1990) and LTP mediated excess transfer of DG into human LDL particles (Singh *et al.*, 1992a) are analogous to the PL-C induced creation of DG molecules *in situ* on the surface of lipoproteins. Amphipathic apolipoproteins, such as human apo A-I and insect apoLp-III, can stabilize the particles. We therefore hypothesized that the presence of DG in the surface by any means provides the binding sites for amphipathic apolipoproteins.

On the other hand, the decrease of DG content is concomitant with the loss of apoLp-III. In our experiment of the facilitated transfer the DG of HDLp-A to human LDL catalyzed by LTP (Chapter 2; Liu and Ryan, 1991), it has been demonstrated that the nearly complete loss of DG during the transformation from HDLp-A to VHDLp resulted in the dissociation of two apoLp-III molecules from lipophorin particle (see Figure 2.2 of Chapter 2). Same result had been observed *in vivo* during the formation of VHDLp-E in oocyte from hemolymph HDLp-A (Kawooya *et al.*, 1988).

It has been demonstrated that hydrolysis of DG molecules of LDLp into free fatty acids by TG lipase results in formation of HDLp particles with a decreased DG content, a smaller particle size and increased density (Kawooya *et al.*,1991). ApoLp-III, originally associated with LDLp, was diminished after TG lipase treatment. This result is consistent with the present observations in which PL-C treatment of lipophorins induced apoLp-III binding, as well as above phenomenons. Hiraoka *et al.*, however, reported that lipoprotein lipase treated LDLp resulted in 90% DG deletion and no apoLp-III dissociation occured (Hiraoka *et al.*, 1992). More works are needed for clarifying such discrepancy.

PL-A_2 treatments of *M. sexta* HDLp-A and LDLp (Kawooya *et al.*, 1991) or *Rhodnius prolixus* lipophorin (Gondim *et al.*, 1992) in the presence of fatty acid-free albumin (to remove free fatty acids and lysophosphatidylcholine generated by phospholipase A_2 activity), however, did not observe particle destabilization, leading the authors to conclude that phospholipids are not necessary for the stability of lipophorin. In the present experiments, however, conversion of phospholipids to DG induced destabilization of lipophorins. A possible explanation for this apparent discrepancy could be that PL-A_2 product, lysophospholipids, were more water soluble and taken away from lipoprotein particles by BSA. In this case the apolipoprotein frame underwent a structural re-organization by which the empty space used to be occupied by phospholipid cylindria would be squeezed to ensure not being exposed to water. On the other hand, DG molecules after hydrolyzing phospholipids by PL-C would not dissociate from the particle, nor it packed in the center unless the core has more room to accomodate these hydrophobic molecules, like HDLp-W2. If the core does not have more space, like HDLp-W1, HDLp-A and LDLp, the DG molecules produced by PL-C would stay at their original position where a hydrophobic area was generated. Therefore the binding of amphipathic apolipoprotein would be necessary in order to maintain a stable particle. Those experiments clearly demonstrated that apolipoprotein matrix plays a very important, active role to deal with the changes of lipid content. The initial response of such change is to modulate its apoprotein structure to accept more lipid in the core. If there is no more space in the core, hydrophobic lipids on the surface will trigger the binding of amphipathic apolipoprotein(s) present in the plasma or hemolymph. If the core lipid content decreased significantly, such as TG lipase hydrolysis, the dissociation of the excess surface components will be inevitable. This flexible, dynamic frame functions as a reusable vehicle during the metabolic changes of lipid contents *in vivo*.

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Table 8.1. Compositional analysis of four lipophorins treated with PL-C.

	Protein (μg/μl)	PL (μg/μl)	DG (μg/μl)	DG / Protein	PL / Protein
HDLp-W2					
Control	0.29	0.05	0.092	0.317	0.172
PL-C	0.34	<0.01	0.162	0.476	<0.029
HDLp-W1					
Control	0.23	0.05	0.134	0.583	0.217
PL-C	0.40	<0.007	0.353	0.883	<0.018
HDLp-A					
Control	0.52	0.13	0.593	1.140	0.25
PL-C	0.45	<0.008	0.586	1.302	<0.018
LDLp					
Control	0.55	0.12	1.024	1.862	0.22
PL-C	0.45	<0.008	0.847	1.883	<0.018

Data were obtained by the average of duplicate assays. Control incubation contained lipophorin and apoLp-III without PL-C; PL-C incubation contained lipophorin and apoLp-III in the presence of PL-C. After reaction, the samples were subjected to density gradient ultracentrifugation to remove unbound apoLp-III. Lipophorin containing fractions were collected. The protein, phospholipids and glycerolipids were determined according the text.

Table 8.2. Stoichiometry of four lipophorins incubated with apoLp-III in the presence and absence of PL-C

	HDLp-W2		HDLp-W1		HDLp-A		LDLp	
	Ctl	PLC	Ctl	PLC	Ctl	PLC	Ctl	PLC
apoLp-I	1	1	1	1	1	1	1	1
apoLp-II	1	1	1	1	1	1	1	1
apoLp-III	0	0	0	3	2	7	16	20

[a] Data was obtained by scanning apoLp-II and apoLp-III bands of gel shown in Figure 8.3. Ctl denotes Control, in which the lipophorin was incubated with equal amounts of apoLp-III without phospholipase-C. PL-C denotes phospholipase C treated lipophorin in the presence of equal amounts of apoLp-III. After incubation samples were subjected to ultracentrifugation to remove unbound apoLp-III. SDS-PAGE was conducted. apoLp-III bands was scanned using apoLp-II bands as an internal standard.

Figure Legends:

Figure 8.1. Kinetic analysis of phospholipase C induced lipophorin sample turbidity development. Panel A) 300 µg protein of each of four lipophorin subspecies were incubated at 37 °C with and without 2 units of PL-C in buffer (150 mM NaCl, 50 mM Tris-HCl, pH7.5, 2mM Ca^{2+}) . Panel B) 100 µg phospholipid of each of four lipophorins were incubated at 37 °C for the specified times in the presence and absence of PL-C (2 units). Panel C) 300 mg DG of each of four lipophorin subspecies were incubated at 37 °C in the absence and presence of PL-C (2 units). At specified time points sample absorbances were measured at 340 nm and expressed here as relative values of individual lipophorin obtained by substracting the readings of its corresponding control sample without PL-C at time of reading. Open square=HDLp-W2; closed diamond=HDLp-W1; closed square=HDLp-A; open diamond=LDLp.

Fig 8.1

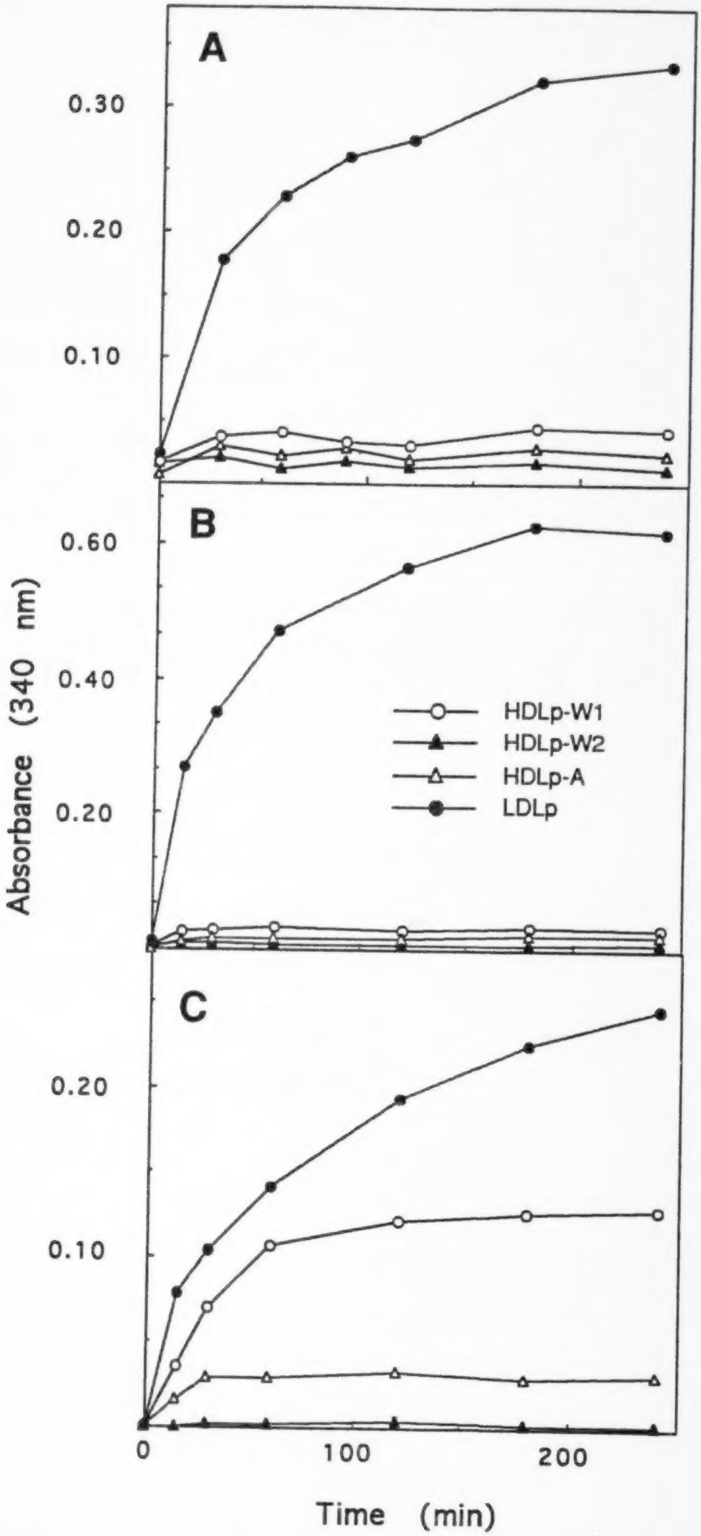
A
0.30
0.20
0.10
B
0.60
0.40
0.20
Absorbance (340 nm)
HDLp-W1
HDLp-W2
HDLp-A
LDLp
C
0.20
0.10
0
100
200
Time (min)

Figure 8.2 Electron microscopy of phospholipase C treated lipophorins. Lipophorin samples were incubated in the absence (panel A) and presence (panels B and C) of phospholipase C (2 units per 300 μg protein) as described in Experimental Procedures. The lipophorin samples were stained with 2% sodium phosphotungstate prior to microscopy. A) HDLp-W2 control; B) HDLp-W2 plus PL-C and C) HDLp-W1 plus PL-C.

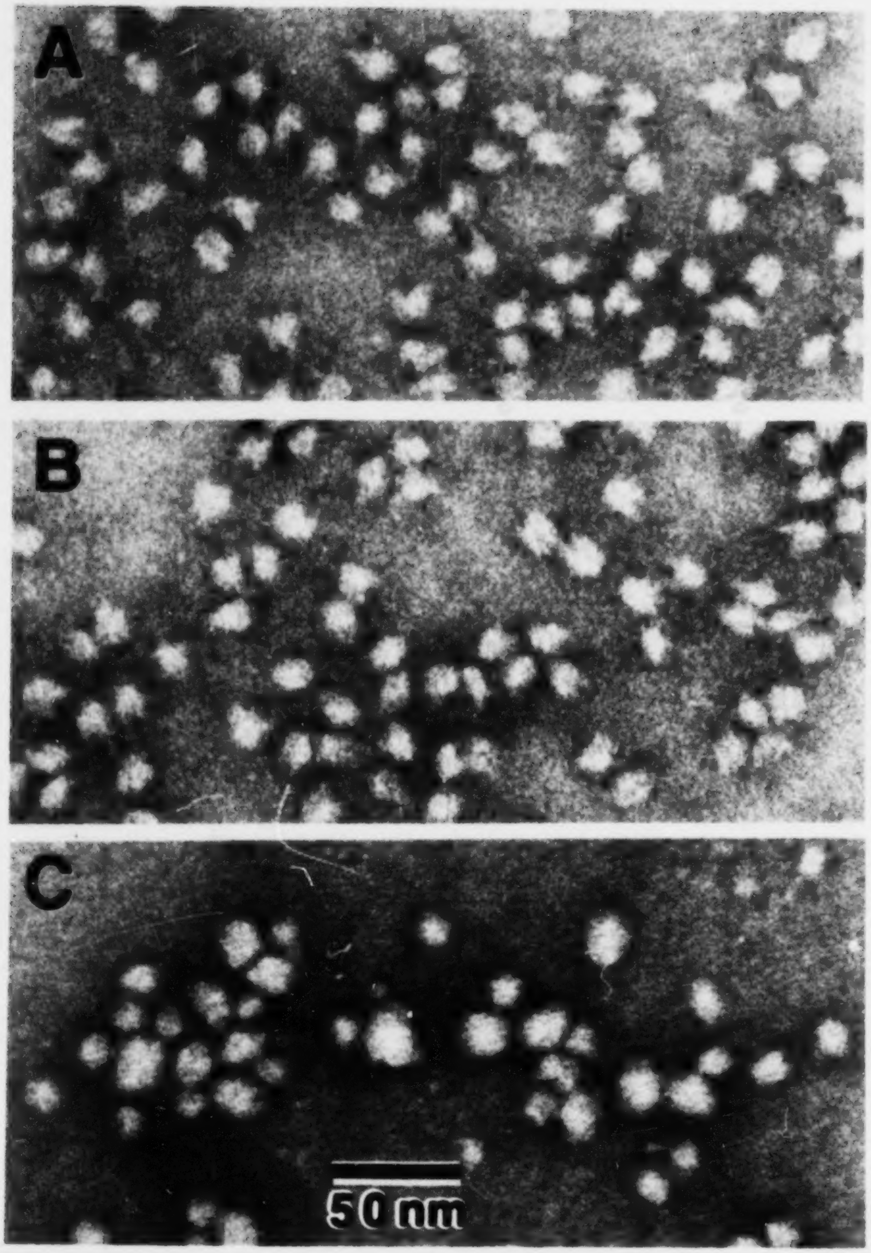

Figure 8.2

Figure 8.3 Protection of PL-C induced lipophorin solution turbidity development by apoLp-III. 1 mg lipophorin subspecies were incubated at 22 ^{o}C with 2 units PL-C in the absence and presence of apoLp-III (1 mg). Relative readings at 7 hours of indivual lipophorin subspecies were expressed. Closed bar=PL-C treated lipophorin with apoLp-III; open bar=PL-C treated lipophorin without apoLp-III; hatched bar=readings of turbid samples obtained by 1 minute mechanically vortexing at maximam setting.

Fig. 8.3

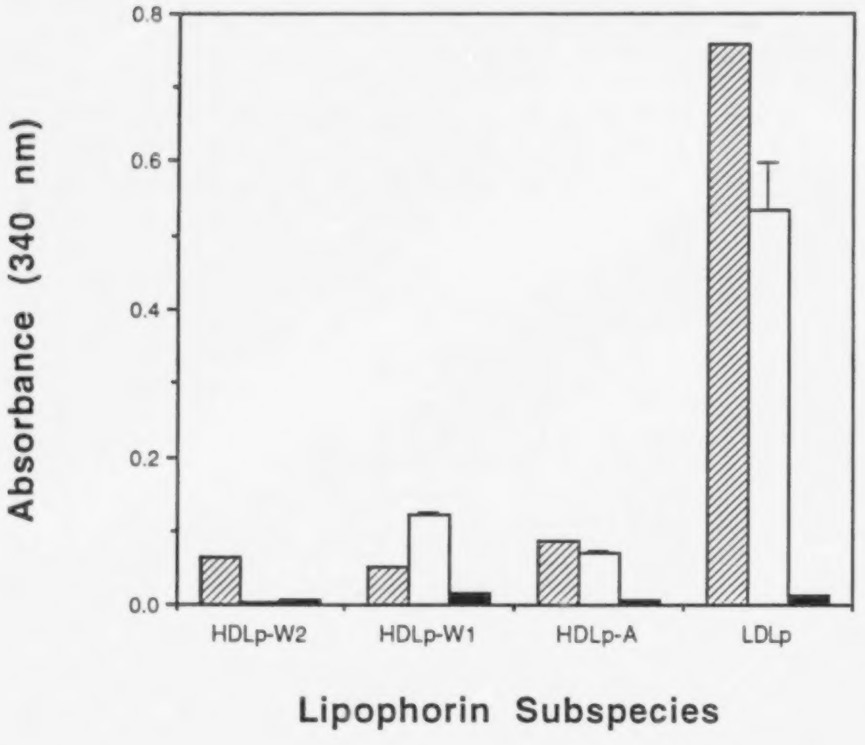
Absorbance (340 nm)
0.8
0.6
0.4
0.2
0.0
HDLp-W2
HDLp-W1
HDLp-A
LDLp
Lipophorin Subspecies

Figure 8.4 Effect of apoLp-III concentration on the solution turbidity of PL-C treated LDLp. LDLp (50 μg phospholipid) was incubated with phospholipase C (1 unit) in the presence of indicated amounts of apoLp-III. A340 values reported were obtained after 4h incubation. Values reported are the average of three measurements ± standard deviation (n=3)

Fig. 8.4

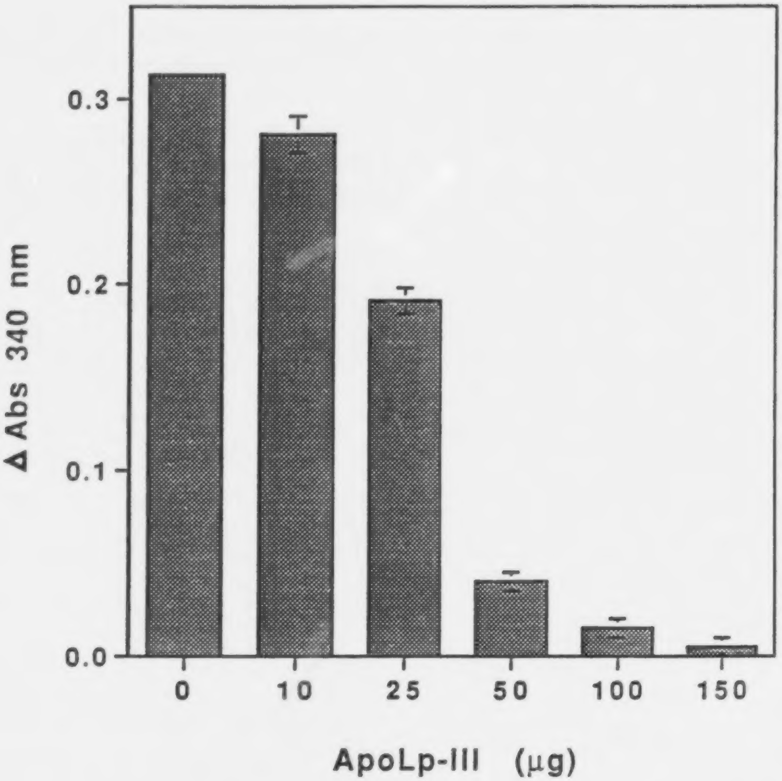

Figure 8.5 Effect of apoLp-III on the morphology of PL-C treated LDLp. LDLp (50 μg phospholipid) was incubated with phospholipase C (1 unit) at 37 °C for 2 h. Following incubation, samples were negatively stained with 2 % sodium phosphotunstate and photogrphed in the electron microscope. A) Untreated control LDLp; B) LDLp plus PL-C (1 unit) and C) same incubation as (B) plus 100 μg apoLp-III.

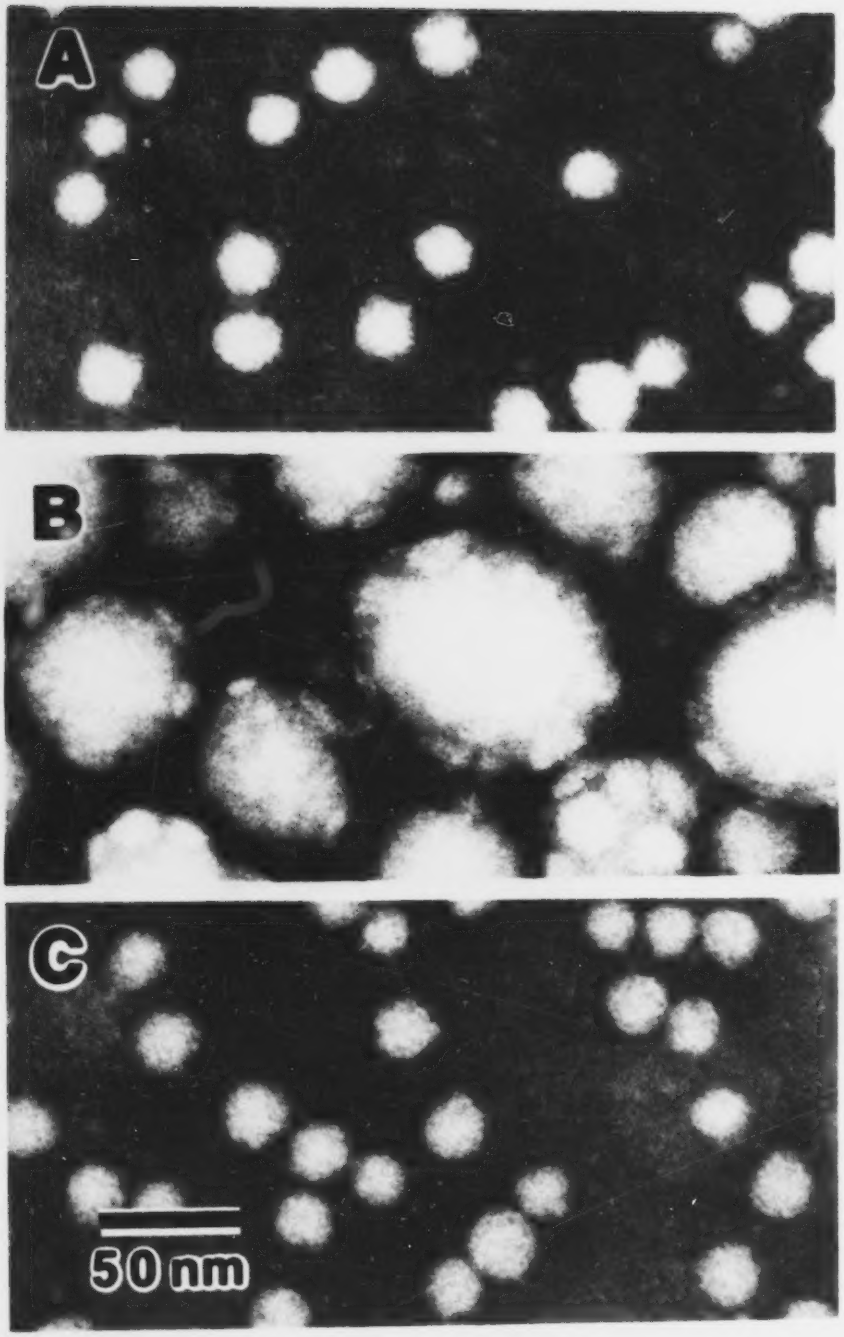

Figure 8.5

Figure 8.6 The effect of phospholipase C on apoLp-III binding to lipophorins. 400 µg each of lipophorins was incubated with 400 µg of apoLp-III in the absence and presence of 8 units of PL-C at 22 °C for 12 hours. Reaction was stopped by adding 100 µl 50 mM EDTA. The samples were brought to a density of 1.21 and subjected to density gradient ultracentrifugation. Top fractions containing lipophorins were subjected to SDS-PAGE. lane 1, 3, 5 and 7 are four lipophorins incubated with apoLp-III in the absence of PL-C in following order: HDLp-W2, HDLp-W1, HDLp-A and LDLp; lane 2, 4, 6 and 8 are the four lipophorins incubated with apoLp-III in the presence of PL-C in following order: HDLp-W2, HDLp-W1, HDLp-A and LDLp; lane 9 is standard protein marks.

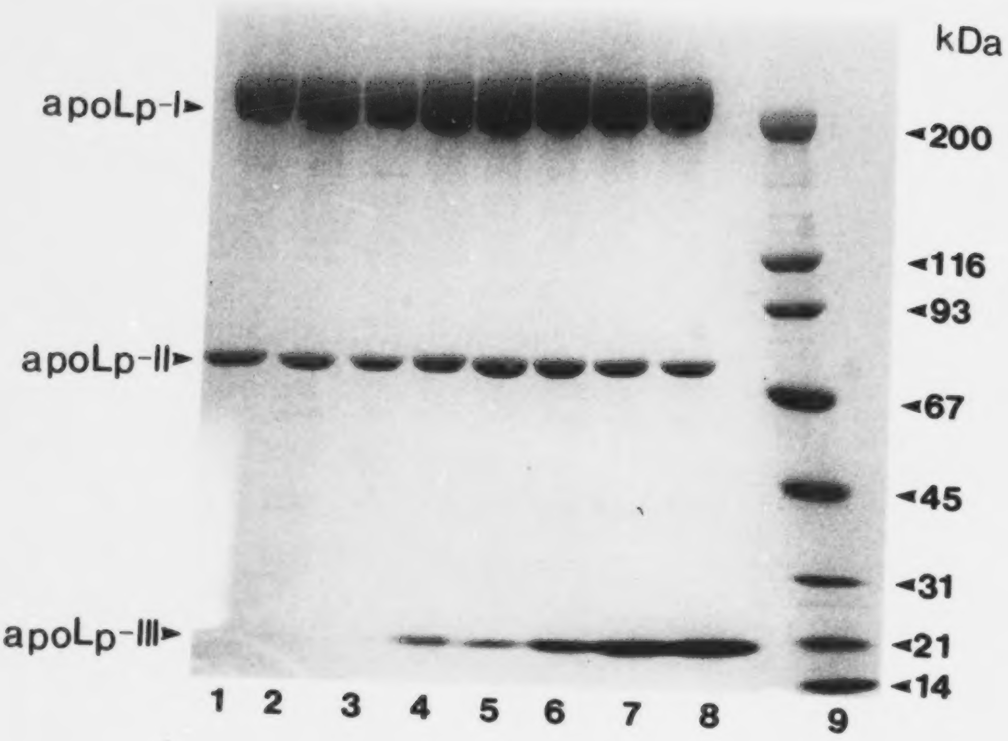
kDa
apoLp-I►
◄200
◄116
◄93
apoLp-II►
◄67
◄45
◄31
apoLp-III►
◄21
◄14
1
2
3
4
5
6
7
8
9

Figure 8.6

Chapter 9

Localization of two distinct microenvironments for the diacylglycerol component of lipophorin particles by ^{13}C-NMR

This is a working draft of a paper for publication: Hu Liu, Jianjun Wang, Brian D. Sykes and Robert O. Ryan. (1993)

Introduction:

NMR spectroscopy has been employed by many investigators to study mammalian lipoproteins. ^{13}C-NMR studies of the lipid moiety of human lipoproteins has provided detailed structural and conformational information. For example it has been shown that unesterified cholesterol molecules in human lipoproteins partition between the surface and core of the particles. Using [4-^{13}C-] cholesterol, ^{13}C-NMR spectra of human low density lipoprotein (LDL) showed that about two thirds of unesterified cholesterol molecules are located on the surface of LDL (Lund-Katz and Phillips, 1986). Like cholesterol in human lipoproteins, diacylglycerol (DG) in insect lipoproteins has been postulated maybe located in the core or at the surface. Direct evidence of such spatial localization, however, has not been demonstrated by any biochemical or biophysical means.

Insect lipoproteins, lipophorins, have similar structural characteristics to human apo B containing lipoproteins. The integral apolipoproteins, apolipophorin-I (apoLp-I) and apolipophorin-II (apoLp-II) have a similar function as human apo B in maintaining a stable particle matrix (Ryan, 1990). On the other hand lipophorins also have unique properties, perhaps most notably the presence of DG, not TG and CE, as their major lipid component. The basic matrix of the lipophorin has the capacity to accept or donate DG, forming different lipophorin subspecies which have distinct structures and morphologies (Ryan *et al.*, 1992). In the adult moth stage the resting form of lipophorin, HDLp-A, contains about 300 DG molecules per particle. During flight large amounts of fat body DG is loaded onto pre-existing HDLp-A particles to form a larger low density lipophorin (LDLp) particle which contains over 1000 DG molecules per particle. Presumably, DG molecules of LDLp can partition between the core and the surface monolayer (Ryan, 1990). Owing to its smaller, less polar head group compared to phospholipids, DG molecules present in the surface monofilm will destabilize the monolayer unless additional surface components are provided (Ryan *et al.*, 1986). Such extra surface binding demands are not met by an increase in phospholipids, which remain constant at about 140 molecules per particle during the HDLp-LDLp transformation (Wang *et al.*, 1992). Rather the DG enriched LDLp particle is stabilized by binding amphipathic apolipophorin-III (apoLp-III) molecules (Ryan, 1990). ApoLp-III is an abundant 18,000 Da hemolymph protein usually found in a lipid free state. It has been shown by X-ray crystallography that in the lipid-free state apoLp-III forms an α-helical bundle organization in which its hydrophobic faces of its amphipathic α helices point inward while the hydrophilic faces project into the water (Breiter *et al.*, 1991). It has been proposed that this helical bundle opens upon the binding to lipid surfaces (Kawooya *et al.*, 1986). High density lipophorin (HDLp) forms isolated at different life stages contain significantly less DG than LDLp. Most HDLps contain little or no apoLp-III molecules. Therefore the majority of DG molecules in HDLps are likely located in the core. A previous study of the phospholipid mobility of lipophorin subspecies by ^{31}P-NMR revealed that the phospholipid phosphate nucleus mobility in LDLp is restricted compared to HDLps. We hypothesized that partitioning of DG molecules onto the surface of LDLp promotes the binding of apoLp-III molecules which interact with phospholipids, perhaps via ionic interactions, to constrain their movement (Wang *et al.*, 1992). To test the hypothesis that apoLp-III binding during the transformation from HDLp-A to LDLp *in vivo* is induced by partitioning of DG molecules into the surface monolayer, we treated human LDL (Liu *et al.*, 1993a) and insect lipophorins (Liu *et al.*, 1993b) with phospholipase C (PL-C). Phospholipids are located solely in the surface of human lipoproteins (Shen *et al.*, 1977) and lipophorins (Katagari, 1984). PL-C treatment converts surface phospholipids into DG *in situ*. Without providing extra surface components, PL-C digested LDL or lipophorin particles form aggregates. This aggregation process, however, can be prevented by additional binding of amphipathic apolipoproteins, such as apoLp-III (Liu *et al.*, 1993a and b).

DG molecules present in the surface monolayer, derived from either partitioning or phospholipid conversion *in situ*, will experience a different microenvironment compared to those located in the core of lipophorin particle. The carbonyl carbons of DG present on the surface will be hydrated and interact with other surface components. By contrast core DG molecules will be excluded from exposure to aqueous medium and will exist in an environments to that provided by non-polar organic solvents. The hydrated surface carbonyl groups of DG might exhibit a distinct resonance from that of the non-hydrated core carbonyls of DG. Therefore monitoring ^{13}C-NMR spectra of the carbonyl carbon of DG in lipophorins, either native LDLp or apoLp-III stabilized HDLps after PL-C digestion, may permit us to detect different microenvironments of the core and surface DG molecules and provide information on the relative distribution of this lipid in various lipophorin subspecies.

Materials and Methods:

Materials. Phospholipase-C from *Bacillus cereus* (Grade I; <0.05% sphingomyelinase activity) was purchased from Boehringer Mannheim, Laval, Quebec, Canada; Glyceryl tri(oleate-[1-^{13}C]) was purchased from MSD Isotopes, Poite Clarire Dorval, Quebec, Canada. Linolenic acid methyl ester was obtained from Sigma Chemical Company, St. Louis, MO, USA. Larval and adult *Manduca sexta* were obtained from a continuing laboratory colony reared on a wheat germ based diet according to Prasad *et al.* (1986). Lipophorins were isolated from freshly collected hemolymph of larval or adult *M. sexta* by density gradient ultracentrifugation (Shapiro *et al.*, 1984). HDLp-W2 and HDLp-W1 were isolated from prepupal larvae as described by Prasad *et al.* (1986). HDLp-A and LDLp were isolated from 1-day-old adult moths according to Ryan *et al.* (1986). In order to ensure homogeneous lipophorin preparations, samples were centrifuged a second time. ApoLp-III was purified from adult moth hemolymph as described by Wells *et al.* (1985).

Protein content was determined with the bicinchoninic acid assay (Pierce Chemical Co.), using bovine serum albumin as standard. SDS-PAGE was performed in 4-20 % acrylamide gradient slab gels and stained with Coomassie brilliant blue.

Labeling ^{13}C-HDLp-L: The lipids of regular laboratory diet (from United States Biochemical, Cleveland, Ohio) were extracted by $CHCl_3$/methanol (3:1) in Soxhlet extraction apparatus for over night. The fat-free diet was dried in a fume hood for 24 hours and kept in the dark. Prior to experiments the lipid extracted diet was mixed with 10 mg of cholesterol and 10 mg of linolenic acid methyl ester per 100g of diet. Fat-free diet was prepared according to conventional methods (Prasad *et al.*, 1986). The mid third instar larvae were chosen and shifted from regular diet feeding status to fat-free diet. Before those caterpillars fed fat-free diet stopped to eat, a bolus of 45 µl of glyceryl tri(oleate-[1-^{13}C]) oil was delivered to the mid gut through a plastic microtube. Five hours later the caterpillars were bled and lipophorin was isolated by density gradient ultracentrifugation.

^{13}C-NMR studies. Before the ^{13}C-NMR experiments, lipophorin samples were dialyzed against 50 mM phosphate buffer, pH 7.4, 150 mM NaCl, 1 mM Ca^{++} for at least 24 hours. Lipophorin sample concentrations are indicated in the figure legends. Fourier transform ^{13}C-NMR experiments were carried at 75.4

MHz with a Varian UNITY 300 spectrometer with proton decoupling. D_2O was used as an internal lock and shim signal. Using a 10 mm NMR tube, 2.5 mL of the lipoprotein sample was mixed with 0.5 mL D_2O at room temperature and placed into the NMR tube. 2,2-Dimethyl-2-silapentane-5-sulfonate (DSS) was used as external chemical shift reference since it is insensitive to temperature and pH variations (Wishart and Sykes, 1993). The ^{13}C-NMR spectra were taken at different temperatures (4 °C, 22.5 °C and 37 °C). Before data were acquired, the samples were equilibrated for at least 30 min at a given temperature. The spectral width was 18000 Hz. All spectra were processed by using the line-broading parameter equal to 1.0 Hz.

For all lipophorin samples, except HDLp-W2, three ^{13}C-NMR spectra were taken at a given temperature, which corresponded to: 1) lipophorin itself; 2) lipophorin with apoLp-III; 3) lipophorin with defined amounts of apoLp-III and phospholipase-C. The first two spectra were used the control spectra. Since the PL-C treated HDLp-W2 sample was stable without apoLp-III, only two spectra were recorded for this species. For the ^{13}C-DG enriched HDLp-L sample, good quality spectra could be obtained after 1000 scans; for those unlabelled (natural abundance) lipophorin samples, 15000 to 30000 scans were needed to obtain a good signal to noise ratio.

Results and Discussion:

Assignment of ^{13}C-NMR spectra of lipophorins. The natural abundance ^{13}C-NMR spectrum of HDLp-W1 contains several well-separated resonances (Figure 9.1). Like apo B of human lipoproteins, the apoprotein moieties of lipophorin particles do not give rise to detectable resonances. Thus all observed resonances were derived from the lipid moiety. Assignment of these resonances was based on the chemical shift values in comparison with corresponding ^{13}C-NMR studies of human lipoproteins (Hamilton and Morissett, 1986). The major resonances observed in HDLp-W1 are 1) carbonyl carbons from DG, phospholipids and free fatty acids (175-180 ppm); 2) unsaturated fatty acyl carbon atoms (130-134 ppm); 3) amino trimethyl carbons from the choline moiety of phosphatidylcholine (54-60 ppm); 4) acyl chain CH_2 carbons (~30 ppm) and 5) methyl carbons (~27 ppm). The carbonyl region of spectra was well separated from other resonances. The chemical shift values of phospholipids and DG in all lipophorin subspecies are listed in Table 9.1. Since the carbonyl carbon is sensitive to hydration, which in turn affects the chemical shift values, we focused on this region of the spectrum.

Spontaneous acyl chain migration in lipophorins In order to examine the carbonyl carbon in detail, the DG moiety of lipophorin was enriched with 1-^{13}C-DG by instilling a bolus of glyceryl tri(oleate-[1-^{13}C]) to the midgut of larvae fed with fat-free diet (Prasad *et al.*, 1986). The fat-free diet fed animals form a very high density lipophorin (VHDLp) which is DG-deficient. When triolein was supplemented, the lipase present in the gut hydrolyzes the triolein into DG and free fatty acid (FFA). DG is then assembled into pre-existing lipophorin particles, shifting their density to form the larval high density lipophorin subspecies, HDLp-L (Prasad *et al.*, 1986; Liu and Ryan, unpublished data). This process may be mediated through the action of a lipid transfer catalyst, LTP (Ryan *et al.*, 1986). The spectrum of ^{13}C-HDLp-L showed that there is a significant increase in peak intensity at the carbonyl carbon region (Figure 9.2). No other groups were enriched since the triolein used was labeled only at the carbonyl carbon of each fatty acyl chain. When the carbonyl carbon region

(175-180 ppm) was expanded (Figure 9.3), we found three resonances. The smallest one was assigned as free fatty acid derived from the hydrolysis of TG. No specific labeling was found in the phospholipid region. This was consistent with the fact that no specific radioactivity was recovered in the phospholipid moiety after instilling 1-^{14}C-trioleate into the gut of animals fed a fat-free diet (Prasad *et al.*, 1986). DG has two resonances which represent *sn*-1, 2- and *sn*-1, 3-isomers. Lipid analysis demonstrated that newly produced lipophorin was predominantly as *sn*-1, 2-DG form. However, there is a spontaneous acyl chain migration of DG from *sn*-2 to *sn*-3 position during sample storage or lipophorin being subjected to increased temperature, such as 37 °C used in the experiment (Van der Horst, 1990; Hamilton *et al.* 1991; Kodali *et al.*, 1990).

Since HDLp-L contains relatively small amounts of DG, both *sn*-1, 2- and *sn*-1, 3-DG of ^{13}C-HDLp-L are likely localized in a homogeneous core environment and are not hydrated. Therefore we hypothesized that the presence of a more complicated resonance pattern in DG-enriched lipophorin subspecies may suggest the presence of heterogeneous environments of DG molecules.

Natural abundance ^{13}C-NMR spectra of DG-enriched lipophorin-LDLp. Natural abundance ^{13}C-NMR spectra of native LDLp reveals a complex pattern, compared to HDLp-L (Figure 9.4). At 5 °C slow particle motion results in broad line widths. As the temperature is increased to 22.5 and 37 °C, however, the spectra exhibited five well-separated resonances. Chemical shift values for phospholipids, *sn*-1, 2- and *sn*-1, 3-DG changes as a linear correlation to the temperature, indicating the validity of experiment. Such chemical shift value changes were also found in the dilauroylglycerol incorporated phospholipid bilayer systems (Hamilton *et al.*, 1991). By comparing the expanded carbonyl region of native LDLp spectrum with that of ^{13}C-HDLp-L, both obtained at 37 °C, we observed that the first 2 resonances of LDLp from upfield dispaly similar chemical shift values to that from *sn*-1, 3-DG in ^{13}C-HDLp-L and the next two resonances of LDLp have similar chemical shift values to that of *sn*-1, 2-DG in ^{13}C-HDLp-L. Since *sn*-1, 3-DG molecule is more symmetric and less polar than *sn*-1, 2-DG, the majority of more compacted *sn*-1, 3-DG molecules may reside in the core of LDLp particle. The occurrence of two resonances of both *sn*-1, 2- and *sn*-1, 3-DG might be due to 1) the dietary intake of both saturated and unsaturated fatty acids or; 2) alternatively, the DG molecules being located in the same environments as the neat *sn*-1, 2-dilauroylglycerol and the *sn*-1, 2-dilauroylglycerol in organic solvents, whose spectra showed two well-separated carbonyl resonances (Hamilton *et al.*, 1991). The chemical shift values in the upfield range also suggest that these DG are located in the non-polar environments. (Hamilton *et al.*., 1991)

The single resonance located in the most downfield region is distinct from the other four (175.53 ppm). We assigned this one as *sn*-1, 2-DG molecules present on the surface of LDLp. Hydrogen bond formation of the carbonyl oxygen with H_2O will deshield the carbon nucleus causing it to resonate downfield (Hamilton el. *al.*, 1991). ^{13}C-NMR study of the interfacial conformation of DG in phospholipid bilayers demonstrated that since the carbonyls have a location within the polar interface, the DG molecules must have a general orientation similar to that of the phospholipid molecules, *i.e.* the acyl chains are roughly parallel to the acyl chains of phospholipid and the DG fatty acyl methyls are in the bilayer interior (Hamilton *et al.*, 1991). This conclusion is similar to the results of molecular modeling of DG molecules between phospholipid molecules in the monolayer of lipophorin particles based on our ^{31}P-NMR study (Wang *et al.*, 1992). As discussed earlier ^{31}P-NMR of lipophorins demonstrated that the monolayer movement of the phospholipds in LDLp was much more restricted than that of HDLps. The acyl chains of DG molecules may partition into the surface of LDLp and interact with the acyl chains of surface phospholipids. The smaller and much more hydrophobic head of DG promotes the association of apoLp-III molecules. The polar side groups of apoLp-III will interact with phospholipids to restrict the movement of phospholipids (Wang *et al.*, 1992). In differential scanning calorimetry (DSC) studies of apoLp-III with various charged phospholipid bilayer membranes, it was suggested that such ionic interactions may be a dominant force responsible for initiation of binding (Zhang *et al.*, 1993). Although the natural substrate of apoLp-III binding has been found to be DG, it can also bind other hydrophobic regions. Through a possible re-arangement of salt bridges, lipid-free apoLp-III with an α-helical bundle structure (Breiter *et al.*, 1991) will unfold into an extended conformation to bind the acyl chain region of the phospholipids (Kawooya *et al.*, 1986; Zhang *et al.*, 1993). In LDLp it is plausible that the carbonyl groups of DG molecules in the surface will form hydrogen bonds with water, the polar head groups of phospholipids, polar side chains of apoLp-III and possibly apoLp-I and -II as well, giving rise a distinct resonance which is different from those DG molecules located in the hydrophobic core of LDLp particle.

The ^{13}C-NMR resonances of carbonyl carbons of DG converted from surface phospholipids by PL-C treatment. To confirm that the downfield resonance observed in LDLp truly represents DG molecules partitioned into the surface monolayer, we conducted the ^{13}C-NMR measurements of high density lipophorins before and after conversion of phospholipid into DG by PL-C hydrolysis (Figure 9.5 and 9.6). In most cases, PL-C treated particles become turbid in the absence of apoLp-III. In the presence of apoLp-III they maintain a stable structure, suggesting an interaction. Since the DG molecules originaly associated with HDLps are primarily located in the core of the particles, PL-C induced DG, therefore, must stay on the surface. This fact was consistent with experiments with limited PL-C hydrolysis of a small proportion of the model membrane by incorporating a small amount of ester phospholipid into a nonhydrolyzable ether-linked phospholipid matrix (Bhamidipati and Hamilton, 1993). 1, 2-DG, the product of PL-C hydrolysis, remained associated with the membrane bilayer and did not alter bilayer structure in any detectable way. From our studies of the effect of PL-C hydrolysis on the structural stability of human LDL (Liu *et al.*, 1993a) and four insect lipophorins (Liu *et al.*, 1993b), the DG molecules, converted from surface phospholipids *in situ*, will remain on the surface of lipoprotein particles. In all cases, except HDLp-W2, apoLp-III binding is associated with PL-C hydrolysis. In Figure 9.5 (panel A), ^{13}C-NMR spectra of native HDLp-W2 exhibited two groups of carbonyl resonances with nearly equal intensity, which reflects the relative amount of phospholipids and DG present in this subspecies of lipophorin (Wang *et al.*, 1992). After complete hydrolysis, the resonances of phospholipids disappeared (Figure 9.5, panel B). The newly formed DG exhibits a new resonance located upfield of the original DG resonance. It is interesting to note that PL-C hydrolysis of HDLp-W2 did not induce binding of apoLp-III (see Chapter 8, Liu *et al.*, 1993b). The finding that most newly generated DG still on the surface by ^{13}C-NMR is consistent our previous observation that no gross morphological change can be seen by electron microscopy (see Chapter 8, Liu *et al.*, 1993b). For PL-C treated HDLp-W1, the presence of apoLp-III is necessary in order to obtain stable discrete particles. After hydrolysis DG derived from phospholipids resonate in downfield of the original DG peaks, representing a surface population (Figure 9.6). Similar results were obtained for HDLp-A (data not shown). After PL-C hydrolysis, we could expect that all HDLps will possess two different populations of DG, i.e. intrinsic core DG and newly formed DG from surface phospholipid conversion. Indeed PL-C treated HDLps have a similar resonance pattern as that of native LDLp (Figure 9.7), indicating the existence of surface DG in the LDLp particle. The DG in the core behaves as though they are dissolved in the non-polar organic solvents. The carbonyl carbons in such apolar environments were the most shielded. Therefore they will resonate at upfield and exhibit two well separated peaks because of the existence of two chemically inequivalent *sn*-1 and *sn*-2 carbonyls (Hamilton *et al.*, 1991)

Conclusion: The direct evidence to distinguish the surface and core DG molecules in lipophorin particles is observed by minitoring the carbonyl region of ^{13}C-NMR spectra of lipophorin subspecies. Most DG molecules in HDLps are located in the core of lipophorin particles. The carboyl carbons of these DG resonate in upfield of the spectra. In LDLp particle some DG molecules partition in the surface of particles and interact with apoLp-III, giving rise an unique resonance. Such binding mode is consistent with our previous molecular modelling study and is supported by *in situ* hydrolytic conversion of surface phospholipid into DG by PL-C. The resonance patterns of PL-C treated HDLps are similar to that of native LDLp.

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Table 9.1 Chemical shift values of ^{13}C-NMR spectra of carbonyl from phospholipids and DG of the lipophorins at 37 °C

	PL	DG
^{13}C-HDLp-L	177.73	175.63 175.55
W1+apoLp-III	175.80	175.28 175.21 175.13
W1+apoLp-III+PLC	N.D.	175.24 175.19 175.14 175.09
W2	175.78	175.26 175.13
W2+PLC	N.D.	175.21 175.11 175.06
A+apoLp-III	175.83	175.27 175.15 175.06
A+apoLp-III+PLC	N.D.	175.27 175.14 175.10
native LDLp	176.31	175.77 175.73 175.63 175.58 175.53

PL = phospholipids; DG = diacylglycerol; W1= HDLp-W1; W2 = HDLp-W2;

A = HDLp-A; N.D. = not detectable

Figure Legends:

Figure 9.1 75.4 MHz ^{13}C-NMR spectrum of HDLp-W1: HDLp-W1 sample containing 170 mg of protein was dialyzed in 50 mM PBS buffer (no EDTA). 0.5 ml D_2O was mixed with lipophorin solution, giving a final voulme of 3 ml.

Fig. 9.1

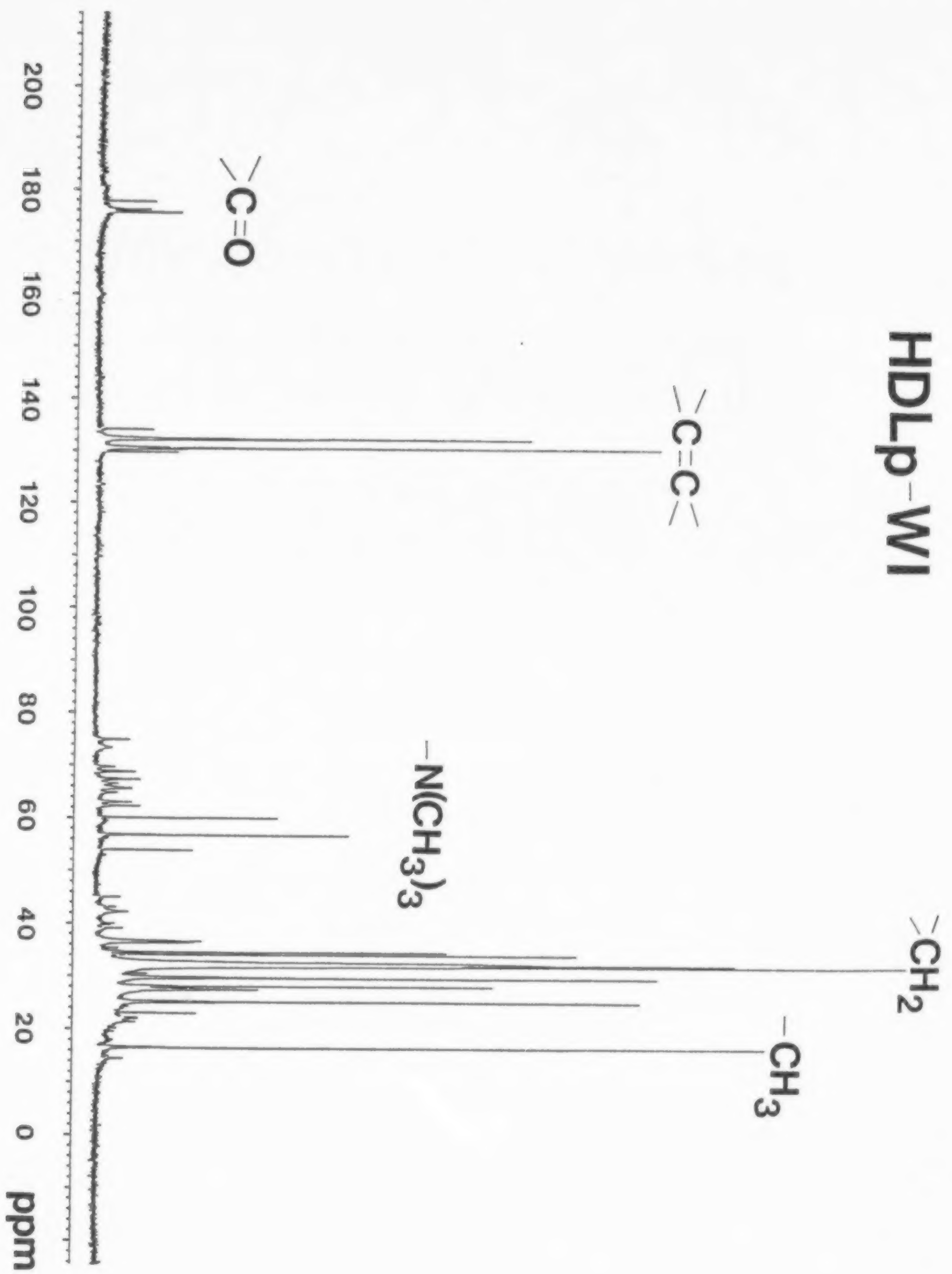

HDLp-WI
>C=O
>C=C<
-N(CH$_3$)$_3$
>CH$_2$
-CH$_3$
200
180
160
140
120
100
80
60
40
20
0
ppm

Figure 9.2 ^{13}C-NMR spectrum of ^{13}C-HDLp-L: 1-^{13}C-HDLp-L sample was obtained by instilling glyceryl-tri(oleate-1-^{13}C) to the mid gut of larvae fed a fat-free diet (see materials and methods). The sample contained 21 mg of protein was dialyzed against 50 mM PBS buffer (no EDTA). 0.5 ml of D_2O was mixed with the solution , giving a final volume of 3 ml.

Fig 9.2

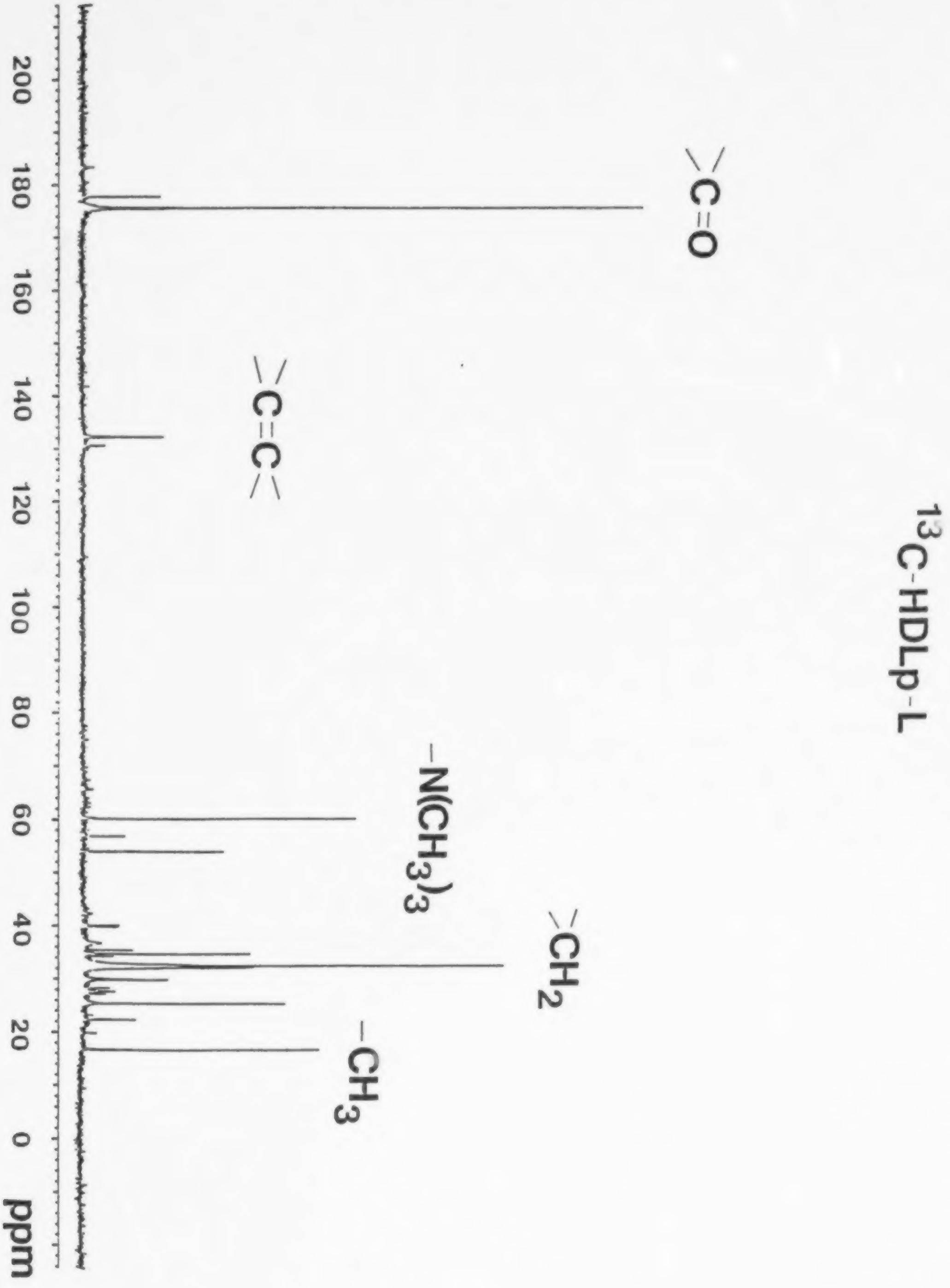

^{13}C-HDLp-L
>C=O
>C=C<
-N(CH$_3$)$_3$
>CH$_2$
-CH$_3$
200
180
160
140
120
100
80
60
40
20
0
ppm

Figure 9.3 ^{13}C-NMR spectrum (carbonyl region) of ^{13}C-HDLp-L: Same conditions as Figure 9.2.

Fig. 9.3

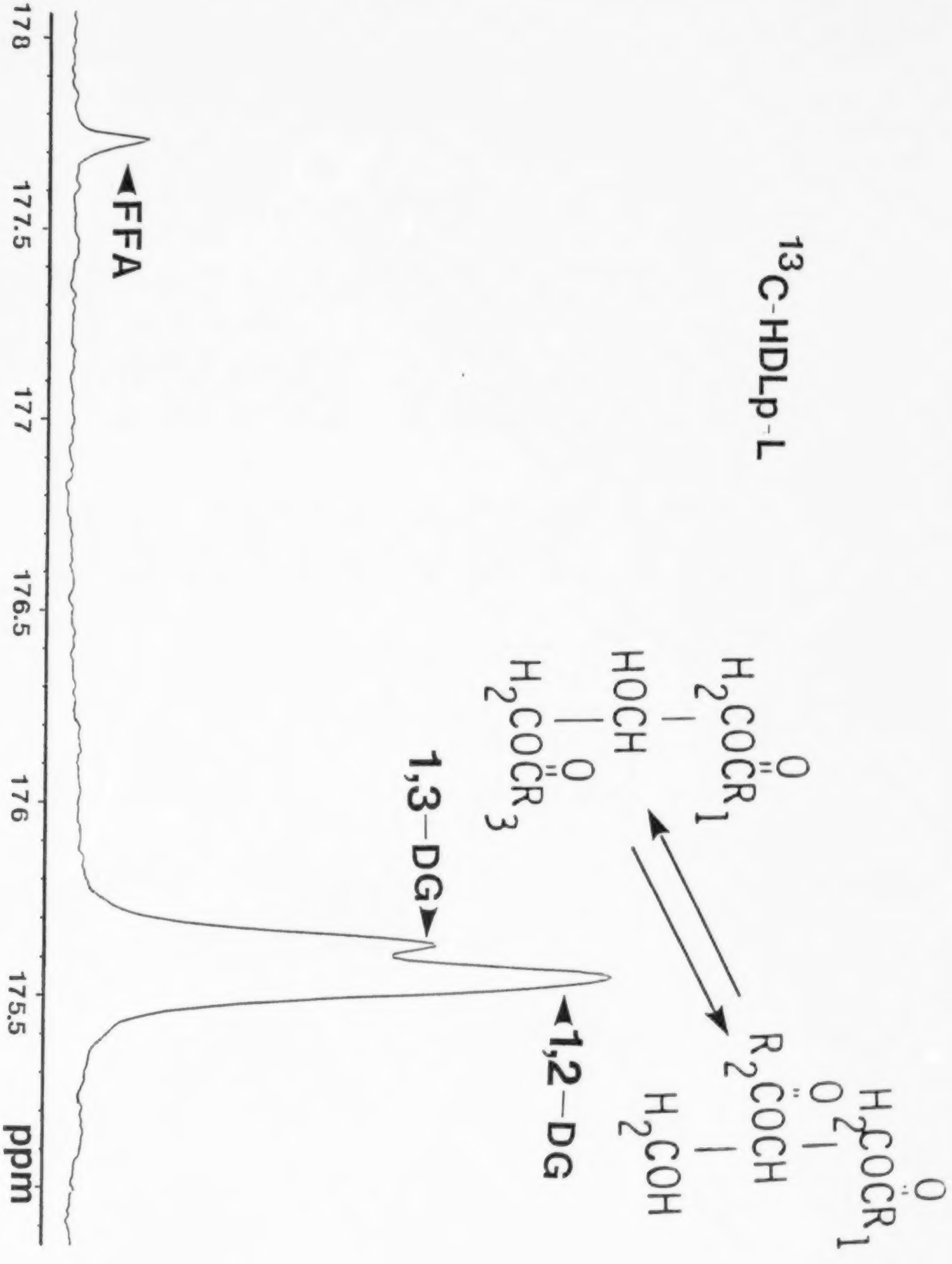


Figure 9.4 ^{13}C-NMR spectrum (carbonyl region) of native LDLp: LDLp sample containing 48 mg of protein was dialyzed against PBS buffer. 0.5 ml D_2O was added to the LDLp solution. The final volume was 3 ml. Panel A: at 5 °C; panel B: at 22.5 °C; panel C: at 37 °C. The insert is a plot of the chemical shift values of *sn*-1, 2-DG (open circle), *sn*-1, 3-DG (open square) and phospholipids (open diamond) as function of temperatures.

Fig 9.4

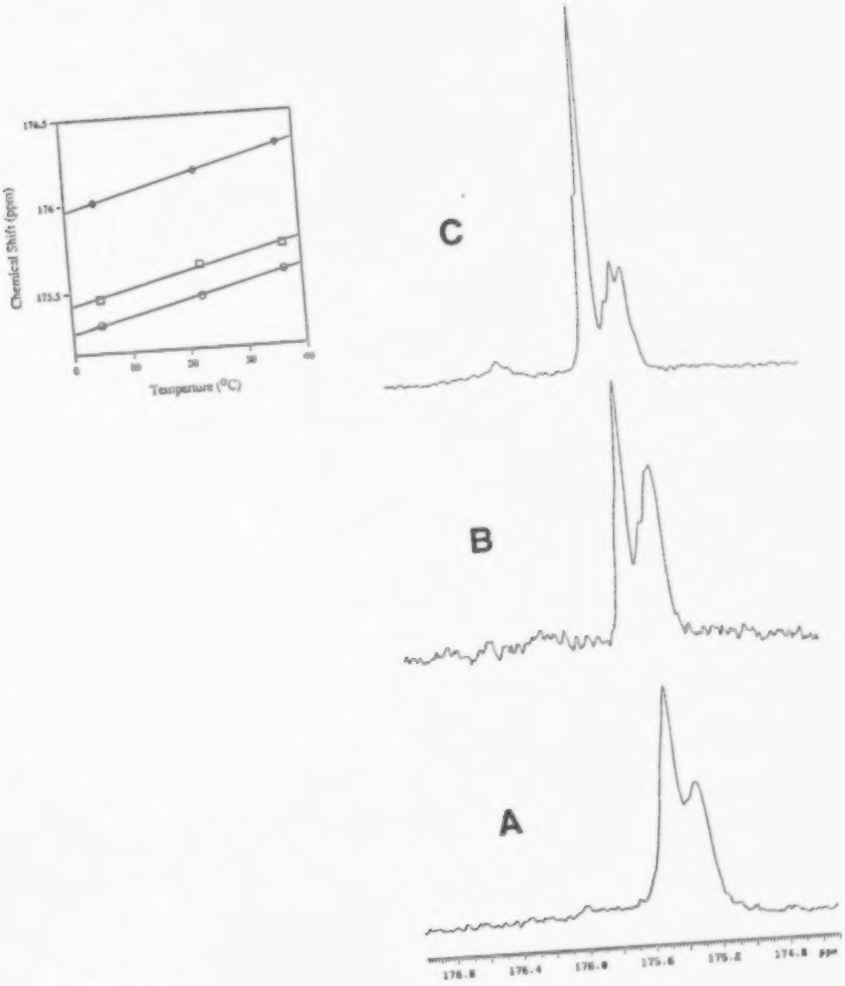

176.5
176
175.5
Chemical Shift (ppm)
0
10
20
30
40
Temperture (°C)
C
B
A
176.8
176.4
176.0
175.6
175.2
174.8
ppm

Figure 9.5 ^{13}C-NMR spectra (carbonyl region) of HDLp-W2 before and after PL-C hydrolysis: A). Control spectrum of HDLp-W2. The sample contained 200 mg of protein was dialyzed against PBS buffer and spectrum was recorded at 37 °C; B) 40 units of phospholipase-C was added to start hydrolysis. 4 hours later, spectrum was recorded.

Fig. 9.5

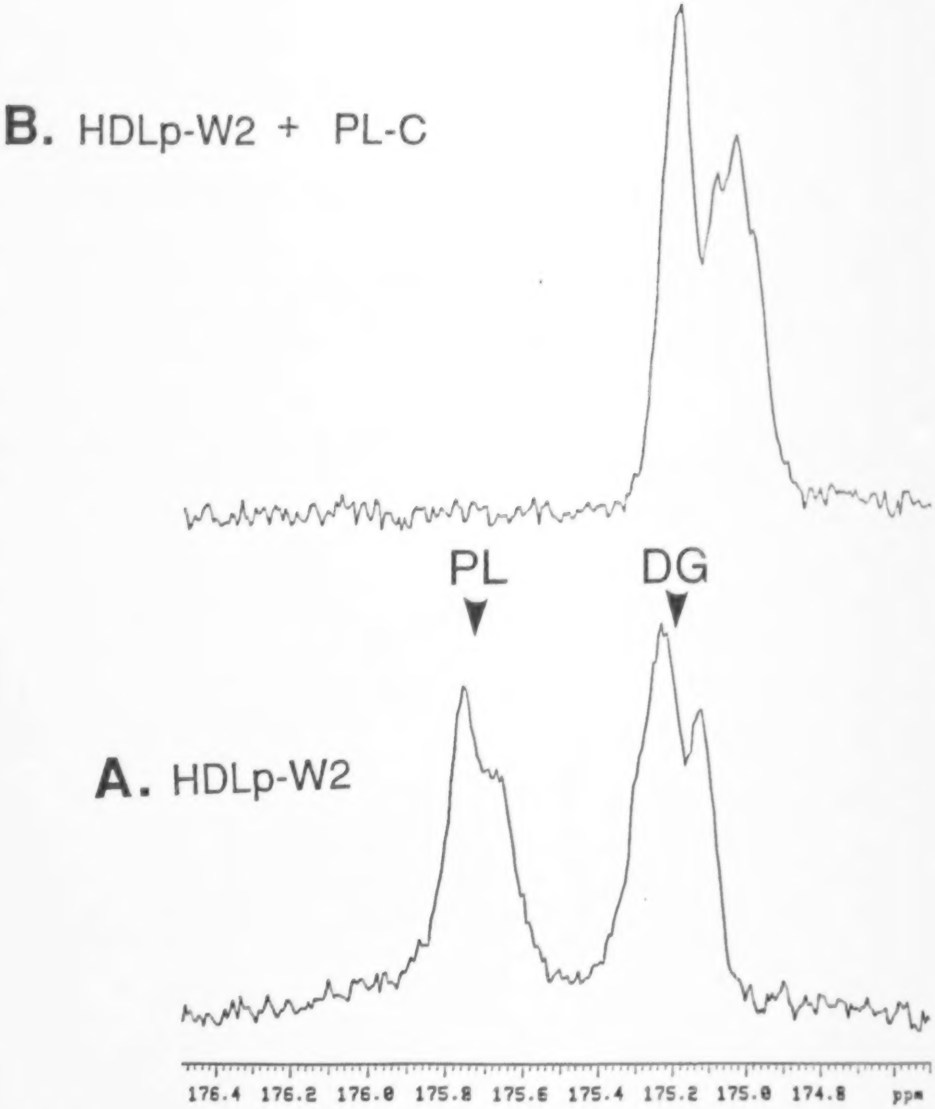

B. HDLp-W2 + PL-C
PL
DG
A. HDLp-W2
176.4
176.2
176.0
175.8
175.6
175.4
175.2
175.0
174.8
ppm

Figure 9.6 ^{13}C-NMR spectra (carbonyl region) of HDLp-W1 before and after PL-C hydrolysis: A). Control spectrum of HDLp-W1. The spectrum of the sample contained 180 mg of HDLp-W1 and 40 mg of apoLp-III was recorded at 37 °C; B) Forty units of phospholipase-C was added to start hydrolysis. 4 hours later, spectrum was recorded.

Fig. 9.6

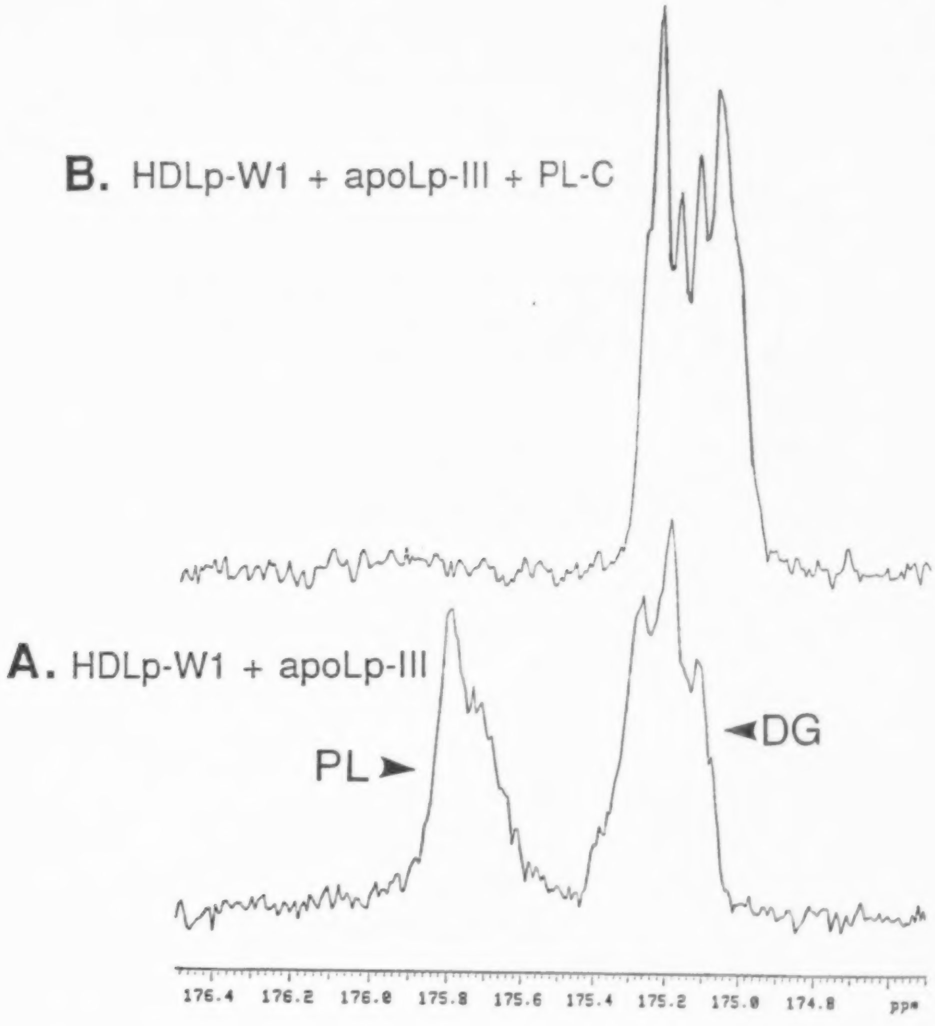
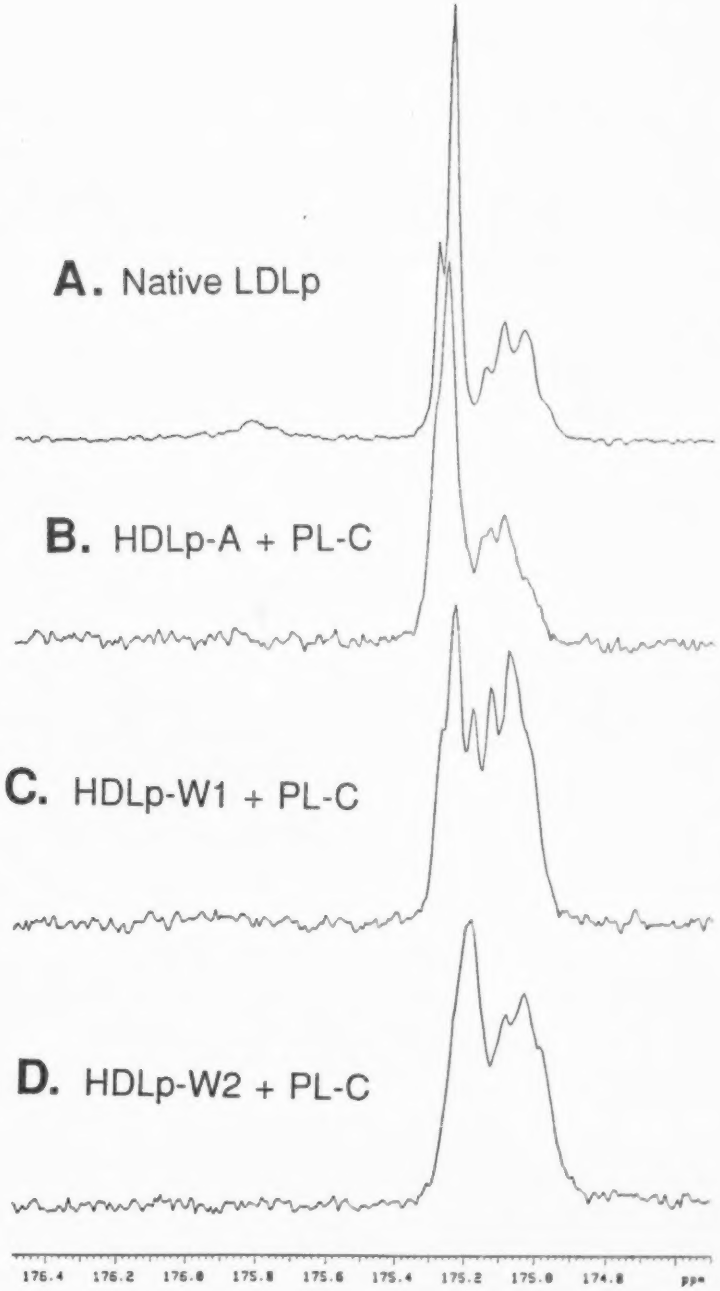

B. HDLp-W1 + apoLp-III + PL-C
A. HDLp-W1 + apoLp-III
PL
DG
176.4
176.2
176.0
175.8
175.6
175.4
175.2
175.0
174.8
ppm

Figure 9.7 ^{13}C-NMR spectral comparison (carbonyl region) of native LDLp with PL-C hydrolyzed HDLps. A). Native LDLp (see condition of Figure 9.3); B). Eighty-one mg of HDLp-A plus 20 mg of apoLp-III had been hydrolyzed by 15 units of phospholipase-C for 4 hours before the spectrum was recorded; C) same conditions as Figure 9.5; D) same conditions as Figure 9.6.

Fig. 9.7


A. Native LDLp
B. HDLp-A + PL-C
C. HDLp-W1 + PL-C
D. HDLp-W2 + PL-C
176.4
176.2
176.0
175.8
175.6
175.4
175.2
175.0
174.8
ppm

Chapter 10.

Discussion and Future Recommendations

The study of this thesis has produced some new perspectives of lipoprotein structure, function and metabolism. Both insect and human lipoproteins are comparatively studied. The overall picture of the two systems are similar but we do find some different aspects. Following is a summary of some key points obtained over the course of study and recommendations for future experiments:

1. Receptor, a missing part of insect oocyte development

LTP is an unique catalyst that mediates net transfer of a variety of hydrophobic molecules. Using an *in vitro* assay system, we established the possible role of LTP in *Manduca sexta* oogenesis in transformation of HDLp-A to VHDLp-E following its uptake from hemolymph. The lipid originally associated with HDLp-A, mainly DG, is removed by LTP present inside oocyte (Chapter 2). One of the remaining questions to be yet addressed is how the HDLp-A particle enters the oocyte. The efficiency and specificity of this transformation suggests that there may be a specific receptor present on the surface of the oocyte. I had explored some preliminary experiments to identify this possible receptor with the help of Dr. Stefano Stefini, then a graduate student in Dr. Wolfgang Schneider's laboratory. After detergent extraction, membrane proteins were separated by SDS-PAGE. Ligand blot experiments demonstrated there were two possible protein bands which may interact with iodinated HDLp-A and chicken plasma vitellogenin (unpublished results), possibly with *M. sexta* vitellogenin which is another important nutrient protein sources for oocyte development. Clearly further experiments are required for the characterization of possible receptor(s) and their substrates. If a receptor(s) is found, a clear pathway of insect oogenesis will be elucidated.

2. Correlation between surface components and core volume of the lipoprotein particles: The third possible way to retailor the smaller, non-apo B containing HDL particles.

It has been demonstrated that several smaller HDL particles can be transformed into larger particles by heat (Tall *et al.*, 1977) or by LTP mediated net lipid transfer (Silver *et al.*, 1990; Chapter 3). Heating HDL particles facilitates the interior core fusion, forming a larger particle. The total surface areas of larger transformed particles are less than that of the substrate HDL particles. The extra surface components, such as apo A-I with relatively low lipid binding affinity, dissociate from the surface.

When apo B like lipoproteins, such as human LDL and insect lipophorins, were treated by phospholipase-C, the lipoprotein particles aggregated (Chapters 7 & 8). When HDL particles were subjected to PL-C hydrolysis, no aggregation occurred (Singh and Ryan, unpublished data, 1993). No aggregation, however, does not mean phospholipids in HDL particles were not converted into DG. It may also form a larger particle since destruction of surface phospholipid can promote the core lipid fusion. When the particle size increases, the total area of the product particles were less than that of the starting particles. Although phospholipid molecules were diminished, the remaining apo A-II and apo A-I molecules might be enough to cover the reduced area of transformed larger particles. It is necessary to test the hypothesis by examining the morphology of PL-C treated HDL under electron microscopy. From those three possible transformation processes, a common scenario emerges that is, the facilitated core lipid interaction and fusion among the HDL particles by heating, diminish amphipathic surface and net transfer result in the formation of larger particles. Therefore the requirement for surface components changes. A stable lipoprotein structure could still be maintained by retaining available amphipathic surface components.

3. *In vivo* functional comparison between human and insect amphipathic apolipoproteins.

We have measured the relative lipid binding affinity of insect apoLp-III. It can be displaced from LDLp particles by human apo A-I (Chapter 4). Although we have demonstrated that apo A-I hybrid LDLp are metabolized *in vivo* over night, it is necessary to test the kinetics of this hybrid LDLp *in vivo* in order to explore the possible role of apoLp-III in activating lipase activity. As discussed earlier, transformation of HDLp-A to LDLp is accompanied by the additional binding of 14 hemolymph apoLp-III molecules. In our displacement experiment, those 14 apoLp-III could be displaced by 7 human apo A-I molecules. It is worthwhile to establish an *in vitro* loading system, in which apoLp-III is substituted by human apo A-I. Therefore we may produce apo A-I hybridized LDLp in such an *in vitro* loading system. In such a system one could ask whether the function of apoLp-III is merely the lipid surface binding or the additional role, such as an activator of lipid loading.

4. Binding mode of apoLp-III to the lipid enriched surface of lipoproteins.

We built a model depicting apoLp-III binding to the DG-enriched LDLp particle (Chapter 6). Subsequent experiments support this model in principle, such as PL-C hydrolysis (Chapters 7 and 8) and ^{13}C-NMR study. Although in the model the α-helical segment of apoLp-III interacts with DG and phospholipids, it is still valid that after all surface phospholipids are converted into DG, the expanded hydrophobic faces of helices patch on the DG-dominant lipoprotein surface. The model, however, was initially constructed based on the fact of restricted motion of phospholipids in LDLp particles. It was built on a plane geometry and did not consider the particle curvature. Therefore one fact was overlooked, i.e. the OH group of glycerol backbone in DG molecule would unrealistically interact with the hydrophobic domain of α helices. This is a thermodynamically unstable situation. In fact this OH group is likely hydrated (Kaiser, E.T. and Kézdy, F.J., 1983 and 1984). Obviously further experiments are required to propose a refined model. Once a good expression system of apoLp-III is set up, the combination of multi-dimensional NMR techniques and site-directed mutagenesis might give some more detailed information of its interaction with lipids.

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